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The expression of the alpha-galactosyl epitope in mammalian tissues

Author
Thall, Aron D.

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The Expression of the Alpha-Galactosyl Epitope in Mammalian Tissues

by

Aron D. Thall

DISSERTATION

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DEDICATION

This dissertation is dedicated in memory of my grandfather, Joseph Thall, who was a constant source of inspiration and who dearly wished to live to see me graduate.

ABSTRACT

Approximately 1% of circulating IgG (anti-Gal) in humans interacts specifically with the carbohydrate epitope, Galα1→3Galβ1→4GlcNAc-R (α–Gal), on mammalian glycoconjugates. This carbohydrate structure is present in abundance on cell surface glycoproteins of New World monkey, prosimian and non-primate mammal cell lines and red blood cells. However, the expression of this structure is normally diminished on cells of Old World Monkeys, apes or man. A variety of studies indirectly suggested that this structure is expressed *in vivo* on some human cells. The objective of this work was to determine the *in vivo* expression of α-Gal epitopes on mammalian cells and the potential for expression of these epitopes on human cells.

In order to detect low numbers of α–Gal epitopes on cells and tissues, we developed a highly sensitive radioimmunoassay (RIA) based on the anti-Gal interaction with this epitope. We initially found that the evolutionary pattern of expression of α-Gal epitopes on *in vivo* secreted glycoproteins is similar to that of cell lines and red blood cells. The RIA assay was also utilized to examine the evolutionary pattern of expression of this epitope on mammalian cell membranes of a model tissue, the thyroid. The same evolutionary pattern of α–Gal epitope expression *in vivo* on thyroid cells was observed with solubilized membrane preparations. This pattern of expression correlated with the presence of the activity of the enzyme which synthesizes these epitopes, the α1-3 galactosyltransferase (α1,3GT), in these cells. When fixed, packed membranes from normal

human as well as Graves' disease tissues were analyzed by the RIA assay, α–Gal structures could then be detected. These structures were also detected on three transformed human cell lines. However, the density of α–Gal structures on these human cells and membranes was 200 to 500 fold less than their nonprimate mammalian counterparts. It is suggested that, in humans and Old World monkeys, the disappearance of α–Gal epitopes on thyroid cells is the result of diminished α1,3GT activity. It is argued that the presence of small amounts of α–Gal epitopes on human cells *in vivo* may be important in the initiation of anti-Gal mediated autoimmune processes.

PUBLISHED PAPERS OR SUBMITTED PAPERS RESULTING FROM WORK IN THIS DISSERTATION

Thall, A. and Galili, U. (1990) Detection of Galα1-3Galβ1-4GlcNAc Residues on Secreted Mammalian Glycoproteins (Thyroglobulin, Fibrinogen, and Immunoglobulin G) As Measured by a Sensitive Solid-Phase Radioimmunoassay. *Biochemistry*. 29: 3959-3965.

Galili, U., Thall, A., Macher, B. (1990) Evolution of the Galα1-3Galβ1-4GlcNAc-R Epitope in Mammals. *Trends in Glycoscience and Glycotechnology*. 2(7): 303-318.

Thall, A., Etienne-Decerf, J., Winand, R., J. and Galili, U. (1990) The α-Galactosyl Epitope on Mammalian Thyroid Cells (*submitted*).

Thall, A., Etienne-Decerf, J., Winand, R., J. and Galili, U. (1990) The α-Galactosyl Epitope on Human Normal and Autoimmune Thyroid Cells: Possible relationship to autoimmunity (*submitted*).

Thall, A, and Galili, U. Aberrant Expression of the α-Galactosyl Epitope on Transformed Human Cell Lines (*in preparation*).

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ABBREVIATIONS

α1,3GT	α1-3 galactosyltransferase
α-Gal epitope	Galα1→3Galβ1→4GlcNAc-R
anti-Gal	Natural anti α-galactosyl IgG antibody
BS lectin	*Bandeiraea simplicifolia* I lectin
BS⁺ thyroglobulin	Thyroglobulin affinity purified on a BS lectin column.
BSA	Bovine serum albumin
CPH	Ceramide pentahexoside
EBV	Epstein-Barr virus
ELISA	Enzyme-Linked immunosorbant assay
FITC	Fluorescein isothiocyanate
Gal	Galactose
GlcNAc	N-acetylglucosamine
HBL	Human blood lymphocytes
HCl	Hydrochloric acid
IgG	Immunoglobulin-G
mAb	Monoclonal antibody
MES	Morpholinoethanesulfonic acid
ND	Not done
N.W.	New World
O.W.	Old World
PBS	Phosphate-buffered saline
RIA	Radioimmunoassay
SA	Sialic acid
SDS-PAGE	Polyacrylamide gel electrophoresis with sodium dodecyl sulfate as detergent
Synsorb 115	Synsorb beads with Galα1→3Galβ1→4GlcNAc conjugates
TBS	Tris-buffered saline
TSH	Thyroid-stimulating hormone
UDP-Gal	Uridine diphosphate-galactose

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CHAPTER I

INTRODUCTION

Background

A large proportion of the outer membrane molecules on eukaryotic cells carry carbohydrate structures. These structures are important for protection of the membrane, cell-cell recognition, and differentiation (1). The terminal carbohydrate structures of glycoconjugates are synthesized within the trans Golgi apparatus (2). A great potential for diversity of carbohydrate structures can be generated due to differences in glycosidic and anomeric linkages. Mammalian glycoproteins and glycolipids contain N-acetyllactosamine units on a large proportion of their carbohydrate side chains. These units may either be abbreviated as LacNAc or represented in chemical nomenclature as Galβ1→4GlcNAc-R. The most common terminal non-reducing sugar linked to this structure is sialic acid (SA) (1). The terminal SA structure is usually SAα2→6Galβ1→4GlcNAc-R or SAα2→3Galβ1→4GlcNAc on glycolipids (3, 4) or glycoprotein carbohydrate chains (4,5). These structures are also found on bi- and multiantennary chains of secreted N-glycosylated mammalian glycoproteins such as thyroglobulin (6, 7, 8), laminin (9), immunoglobulin (10), fibrinogen (11), fetuin (12, 13), erythropoietin (14) and β- and γ-interferon (15). Another carbohydrate structure which has been the subject of increasing interest is the α-galactosyl (α–Gal) epitope with the structure

Galα1→3Galβ1→4GlcNAc-R. As discussed below, this structure is present on a variety of mammalian cells. The study of the α-Gal epitope is of particular interest since as much as 1% of circulating IgG in all humans is directed against this structure. These antibodies have been designated anti-Gal (16).

The Anti-Gal Antibody

Anti-Gal is a natural antibody, i.e. an antibody continuously produced against an antigen. Natural antibodies include those directed against blood group carbohydrate structures, such as anti-A and anti-B antibodies (17, 18). Anti-Gal is an IgG antibody which comprises approximately 1% of the circulating IgG antibodies in healthy humans, independent of blood type (16). As demonstrated below (see Appendix), anti-Gal is produced in much higher amounts than anti-blood group antibodies.

Anti-Gal has been isolated from AB serum by affinity chromatography on melibiose sepharose (Galα1→6Gal) (19) and on synthetic α-Gal epitopes linked to silica beads (20, 21). The specificity of the anti-Gal antibody for the α-Gal epitope has been demonstrated by immunostaining of chemically defined glycolipids on thin layer chromatograms. Anti-Gal showed binding to glycolipids with terminal Galα1→3Galβ1→4GlcNAc structures but not to other structures, such as Galα1→4Gal and fucosyl containing glycolipids. As Table I indicates (reproduced from Ref. 72), glycolipids with closely related structures to the terminal Galα1→3Gal-R did not bind anti-Gal in an immunostaining assay. For example, anti-Gal did not bind to the blood group B glycolipid, B_I, or to the terminal Galα1-4Gal containing glycopids CTH and the human P_1 glycolipid. In the case of

the blood group B glycolipid, no anti-Gal binding would be expected, since the anti-Gal was isolated from blood type AB individuals. The α-Gal epitope was previously reported to be an abundant terminal structure on glycolipids of rabbit erythrocytes (22). In accordance with this finding, anti-Gal was found to readily agglutinate rabbit erythrocytes and this agglutination could be inhibited by α-galactosides, most effectively by Galα1→3Gal structures (23). Therefore, the agglutination of rabbit erythrocytes has been used as an expedient means of determining the activity of anti-Gal in isolates and serum.

The narrow specificity of this interaction was confirmed by other investigators. Towbin et al. (20) and Avila et al., (24) demonstrated this specificity by studying the interaction of anti-Gal in human serum with mouse laminin, a glycoprotein containing between 50 and 70 α-Gal epitopes per molecule (9, 25). The interaction of serum anti-Gal with rabbit red cell glycolipids was also analyzed by thin layer chromatography and shown by Suzuki et al. (26) to interact with a major glycolipid, which contains a terminal α-Gal structure. Studies by Wieslander et al. (27) of the interaction of purified anti-Gal with neoglycoproteins showed that the antibody could interact with Galα1→3Gal structures but not Galα1→4Gal and Galα1→2Gal structures. Recently, the interaction of anti-Gal with α-Gal structures on tissue plasminogen activator (t-PA) has been studied by Tsuji et al. (28). These investigators studied the specificity of the antibody interaction by the ability of synthetic carbohydrates linked to silica beads to adsorb antibody reactivity.

Table I

Anti-Gal Binding to Glycosphingolipids

Name	Source	Structure	Anti-Gal Binding
CPH (ceramide pentahexoside)	Rabbit	Galα1-3Galβ1-4GlcNAcβ1-3-R	+
CHH (ceramide heptahexoside)	Cow	Galα1-3(Galβ1-4GlcNAcβ1-3)$_2$R	+
Glucosyl ceramide	Man	Glc1-1Cer	-
Lactosyl ceramide	Man	Galβ1-4Glc1-1Cer	-
CTH (ceramide trihexoside)	Rabbit	Galα1-4–R	-
Globoside	Man/pig	GalNacβ1-3Galα1-4-R	-
Paragloboside	Man	Galβ1-4GlcNAcβ1-3-R	-
Forssman	Sheep	GalNAcα1–3GalNAcβ1-3Galα1-4-R	-
P_1	Man	Galα1-4Galβ1-4GlcNAcβ1-3-R	-
B_1	Man	Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3-R	-
GM_3	Man	NeuAcα2-3–R	-
Sialylparagloboside	Man	NeuAcα2-3Galβ1-4GlcNAcβ1-3-R	-

R = Galβ1-4Glc1-1Cer

Anti-Gal, although highly specific for the α-Gal structure, is a polyclonal antibody (29) and is produced throughout life. The cause of the constant production of this antibody is thought to be a continuous antigenic stimulation by bacteria of the human gastrointestinal flora (30). Studies of lipopolysaccarides of enteric bacteria, such as Salmonella (31), Klebsiella (32), and E. coli (30), indicated that they contain terminal Galα1→3Gal structures. In addition to man, anti-Gal is also found in large amounts in the serum of apes and Old World monkeys (33). However, it is absent in New World monkeys and nonprimate mammals, which have the capacity for producing the α–Gal epitope (34).

Evolution of the α–Gal epitope in Mammals

The α–Gal epitope was first described as being present in abundance in rabbit red blood cell glycolipids (22). A major neutral glycolipid molecule in rabbit red cells containing the αGal structure was identified as ceramide pentahexoside (CPH) (22, 35). Bi-, tri- and multiantennary polylactosamine chains from neutral glycolipids on rabbit red cells also contain these structures (36, 37). CPH was also found in bovine red cells (38). In addition, bovine red cells were found to contain more complex neutral glycolipids containing α–Gal structures (39), including a biantennary ganglioside (40). Cell lines such as rat hepatoma cells also produce CPH (41). Glycolipids with these α-Gal structures have recently been found in kidney tissues of sheep and pigs (42).

In addition to glycolipids, cells also produce large amounts of glycoproteins containing α–Gal structures. These structures have been characterized as a major carbohydrate epitope on glycoproteins from mouse NIH 3T3 fibroblasts (43), mouse teratocarcinoma cells (44), mouse lymphoma cells (118), bovine thyroid and thymus cells (45, 58), rat mammary carcinoma cells (46) and mouse Ehrlich ascetes cells (47). However, Galili et al. (34) did not find these structures on human cells (34).

Galili et al. have conducted systematic studies to determine the α–Gal expression on red cells and cell lines of a large number of species and observed a striking evolutionary pattern of distribution (33, 34, 48). Whereas cell lines from non-primate mammals and New World monkeys were found to have these structures on their cell surface, these structures were not found on cells of humans, apes or Old World monkeys. Based on paleontologic findings, the Old World monkeys are thought to have diverged from the New World monkeys approximately 35 million years ago (49). Therefore, it would appear that the expression of the α-Gal epitope was suppressed in Old World primates within the last 35 million years.

Hypothetically, this evolutionary phenomenon could have been the result of a geographic isolation between species. Pathogens, endemic to the Old World, which expressed structures similar to the α–Gal epitope might have exerted a selective evolutionary pressure in Old World primates for the production of anti-Gal, as a protective means. Concommitantly, the synthesis of autologous α–Gal epitopes in Old World primates would have been suppressed to prevent autoimmunity. α–Gal epitopes are found today on number of

pathogens, including the Friend murine leukemia virus (50), human gastrointestinal bacteria (30, 31, 32), and protozoa, such as *Leishmania mexicana* and *Trypanosoma cruzi* (51).

As an alternative hypothesis, a toxin affecting target cells via receptors with α–Gal epitopes might have exerted a similar pressure for the suppression of α–Gal epitope expression. It has been shown that enterotoxin A of *Clostridium difficile* displays such specificity (52). The production of anti-Gal antibodies against such a toxin would have led to the loss of immune tolerance to this epitope. The loss of α–Gal epitope expression in man, apes and Old World monkeys appears to be due to the diminished activity of the glycosylation enzyme, α1→3 galactosyltransferase (34).

The α1-3Galactosyltransferase Enzyme

The enzyme which synthesizes the terminal Galα1-3Gal linkage is the N-acetyllactosaminide α1→3galactosyltransferase (α1,3GT). The study of this enzyme is important because, as will be argued, its expression may be involved in human autoimmune diseases. The α1,3GT enzyme synthesizes the terminal Galα1-3Gal epitope by the following reaction:

$$\text{UDP-Gal} + \text{Gal}\beta1\rightarrow4\text{GlcNAc-R} \xrightarrow{Mn^{2+}} \text{Gal}\alpha1\rightarrow3\text{Gal}\beta1\rightarrow4\text{GlcNAc-R} + \text{UDP}$$

(1).

This enzyme is a member of a group of terminal glycosyltransferases which are located in the trans Golgi apparatus (2).

α1,3GT activity has been found in microsomal fractions from mouse Ehrlich ascites cells (53, 54, 55), rabbit bone marrow (56), rabbit stomach mucosa (57), mouse F9 teratocarcinoma cells (52), and bovine thymus (58). The enzyme has been purified to homogeneity from Ehrlich ascetes cells (53) and bovine thymus (58) by affinity chromatography on the acceptor and the substrate. Furthermore, when the α1,3GT activity was analyzed in primate cell lines, Galili et al. found that the enzyme is active in New World monkey cells but is diminished in human cells (34). The lack of α-Gal expression is not due to the lack of acceptors for the α1,3GT because human cells produce LacNAc structures, which are suitable acceptors for the mammalian enzyme (1). When such acceptors were utilized with the bovine α1,3GT, the α-Gal epitope could be synthesized (34). This suggests that the loss of α-Gal expression in humans is the result of an evolutionary suppression of α1,3GT activity.

It is not yet clear whether the absence of α1,3GT activity is the result of mutations in the the structural gene or mutations in the genomic region which regulates the expression of the gene. The α1,3GT cDNA has been cloned by two groups from murine (59) and bovine (60) cDNA libraries. Both groups found homologous sequences within human genomic DNA, implying that the gene has been conserved. Sequencing of the cloned 3' region of the genes revealed a 76% homology between the murine and human genes (62). It is interesting to note that there is almost a 50% homology between the α1,3GT cDNA and the human blood group A and B transferases (62). This suggests that these glycosyltransferases

belong to a gene family. It has also been suggested that the suppression of α1,3GT activity in humans is due to frameshift mutations which might result in a premature termination of the α1,3GT mRNA (62); however, studies by Joziasse et al. (60) support the theory that a mechanism for this suppression could have been mutations which affect the rate of transcription of the α1,3GT gene. These investigators found no α1,3GT mRNA in human or Old World primate cells but an abundance of the mRNA in New World monkey cells. The mechanisms involved in the suppression of the α1,3GT gene remain to be determined. However, in recent years, findings have indicated that the suppression of α-Gal epitope expression is not absolute.

Aberrant Expression of α-Gal Epitopes in Humans

A number of studies have suggested that human cells are capable of producing α-Gal epitopes. These include studies on human carcinoma cell lines (61), senescent erythrocytes (63), vascular and placental tissue plasminogen activator (t-PA) (28). Studies by Castronovo et al. (61) have suggested that the α-Gal epitope may be aberrantly expressed on human tumor cells. Several malignant human cell lines were found to interact with both anti-Gal and *Bandeiraea simplicifolia* B_4 subunit (BS lectin). The BS lectin was shown to have a high affinity for the α-Gal epitope (84). Binding of the lectin and antibody to malignant human cells was inhibited by pretreatment of these cells with α-galactosidase but not β-galactosidase, indicating that this interaction was specific. These

investigators also examined malignant breast tissue specimens and benign lesion specimens for binding of both the lectin and anti-Gal. None of the 24 benign lesion specimens contained cells which interacted with the antibody or lectin. However, 50% of malignant breast tissue specimens contained α–Gal positive cells (61). One might speculate that the anti-Gal interaction with such malignant cells might lead to their immune mediated destruction. These investigators suggested that such an immune reaction may be part of the body's natural defense mechanisms against some malignancies.

In addition to malignant cells, studies by Galili et al. of the interaction of anti-Gal with senescent, dense, human erythrocytes (hrbc) indicated that these cells may also aberrantly express α–Gal structures (19). These studies utilized a highly sensitive and specific method for detecting antibody bound to erythrocyte surfaces, the rosetting antiglobulin test with K562 cells, which bind the IgG on rbc through the F_c portion of the molecule. K562 cells formed 56% rosettes with senescent hrbc versus 5% with young hrbc. Proteolytic treatment of normal hrbc with pronase resulted in an exposure of anti-Gal binding sites. The specificity of this interaction was shown by the abrogation of anti-Gal binding by pretreatment with α-galactosidase but not by β-galactosidase. These epitopes seem to be present in very small numbers on hrbc, at approximately 1,000 structures per cell (63), and thus were not detectable by other means. In addition to anti-Gal interactions with senescent cells, it was also demonstrated that anti-Gal could interact with α-Gal epitopes on a large proportion of pathological hrbc such as thalassemic rbc (66) and sickle rbc (65). Furthermore, the binding of

anti-Gal to sickle rbc and senescent hrbc was shown to mediate phagocytosis of these cells by macrophages *in vitro*. Therefore, Galili et al. (65) suggested that the interaction of anti-Gal with α-Gal epitopes on senescent and pathological hrbc *in vivo* may be part of a physiologic process for the removal of such cells by phagocytes of the reticuloendothelial system.

α-Gal epitopes may also be present in normal human tissues other than rbc in a cryptic form. Immunohistochemical staining of trypsinized normal thyroid epithelial cells with anti-Gal suggests that the antibody can interact these cells. No staining was seen with untreated cells (21). This interaction seems to be specific, since it was eliminated by treatment of the cells with α-galactosidase. The presence of these epitopes on human cells may be a result of marginal α1,3GT activity. Alternatively, these structures may arise as a result of the activity of other glycosyltransferases which are "promiscuous", in that they are capable of recognizing more than one acceptor or substrate. An example of such an enzyme is the fucosyltransferase, recently cloned by Kukowska-Latallo et al. (109), which is capable of synthesizing both fucose α(1,3)- and α(1,4) linkages.

In normal tissues, the number of α-Gal epitopes might not be sufficient to generate an anti-Gal mediated immune response, since these epitopes are present in low numbers, possibly in a cryptic form. In addition, the presentation of the α-Gal epitopes in human tissues is not sufficient for the development of immunological tolerance against these epitopes, as indicated by the fact that 1% of circulating human IgG recognize this epitope. Moreover, it appears

that humans are constantly immunized against α–Gal epitopes by gastrointestinal bacterial flora, which as already mentioned, carry similar structures (30, 31, 32). Therefore, if these epitopes are present in human tissues they may not evoke an autoimmune response unless their density is increased or they are somehow exposed to anti-Gal. Hypothetically, the interaction of α-Gal epitopes with anti-Gal might occur as a result of an aberrant increase in α-1,3GT activity, by exposure of cryptic epitopes, as was proposed for human rbc aging and pathology (65), or perhaps by a clustering of these epitopes on cells surfaces. As Galili et al, suggested (65), anti-Gal might then bind to these structures in sufficient amounts to interact with the Fc receptors of macrophages and granulocytes. It was demonstrated by Galili et al. (65) that blood mononuclear cells can recognize anti-Gal bound to cells and mediate antibody dependent cytolysis. *In vivo*, these interactions could initiate a local inflammatory response. Since inflammation is known to damage physiological barriers to the humoral immune system, normally sequestered antigens may cause the generation of a secondary immune response in which antibodies to tissue specific epitopes would be produced. Thus, the primary interaction of anti-Gal with α-Gal epitopes in human tissues might ultimately lead to autoimmune diseases.

Studies by Etienne-Decerf et al. (21, 69) suggested the involvement of anti-Gal in Graves' disease. Anti-Gal titers were found to be significantly elevated in patients with this disease. Graves' disease is an autoimmune disease of the thyroid, which is characterized by a marked hypertrophy and hyperplasty of thyroid

cells. Thyroglobulin containing colloid is usually completely lost from the follicles. In some patients with long standing Graves' disease, there is also extensive lymphocytic infiltration of the thyroid parenchyma. The Graves' disease thyroid functions at a greatly accelerated pace. The reason for the increased thyroid functioning in this disease is thought to be due to the presence of antibodies directed against the thyroid stimulating hormone receptor.

In addition to immune related diseases of the thyroid, anti-Gal titers may also be elevated in juvenile rheumatoid arthritis patients (67). Another study suggested that anti-Gal levels may increase in children with Henoch-Schoenlein purpura or IgA nephropathy (68). One of the hallmarks of these diseases is the presence of circulating IgA containing immune complexes. These immune complexes are deposited in the glomerular mesangium were they may cause nephritis. Whereas anti-Gal purified from serum is almost exclusively IgG, children with Henoch-Schoenlein purpura or IgA nephropathy had both anti-Gal IgG and IgA (67). The significance of elevated anti-Gal titers in these autoimmune diseases remains uncertain. However, one might speculate that the increase in antibody titers is due to an aberrant expression of the α-Gal epitope in the diseased tissues.

Objectives

The principal objectives of this dissertation project are as follows: 1) to develop a sensitive quantitative technique for studying the expression of low numbers of α-Gal epitopes on cells and tissues;

2) to explore whether the evolutionary pattern of expression of α-Gal epitopes seen in cell lines reflects the pattern *in vivo*; 3) to analyze the possible aberrant expression of α-Gal epitopes on human cell lines and tissues and study the enzyme involved in their synthesis; 4) as an addendum, to observe whether the high level of anti-Gal in serum is reflected in a high proportion of circulating B lymphocytes capable of secreting this antibody.

The first objective is important because preliminary data suggests that if the α-Gal structure is present in humans, it is present in much lower quantities than in non-primate mammals. Therefore, the technique developed would have to be sufficiently sensitive to detect very low α-Gal epitope numbers. In order to compare the expression of these epitopes on different cells, it would also be desirable that this technique be quantitative.

It is possible that the expression of α-Gal epitopes on cell lines might not reflect the expression of these epitopes *in vivo*. Therefore, the evolutionary pattern of expression of these epitopes will be analyzed on secreted proteins and tissues from a variety of mammals including humans. These studies will be important to confirm the evolutionary suppression of α-Gal epitope synthesis in man.

The finding that senescent and pathological human red blood cells may have exposed αGal epitopes posed the question of whether α-Gal epitopes might be expressed on nucleated cells under abnormal circumstances. As a means to address this issue, human cell lines will be examined for the aberrant expression of these epitopes. Several parameters will be explored in these studies. One study will probe a variety of human cell lines for the presence of α–Gal

epitopes. Another issue which will be addressed, if human cells are found to express these epitopes, is the biological outcome of the binding of anti-Gal to cells in culture. This will be evaluated by the use of an antibody dependent cytotoxicity assay utilizing normal human lymphocytes

Studies have suggested that anti-Gal levels may be elevated in human autoimmune diseases. As previously mentioned, there is a striking increase in anti-Gal titers in patients with Grave's Disease. To address this issue, thyroid tissues from normal individuals and patients with Grave's Disease will be examined for elevated levels of α-Gal epitopes and the corresponding α1,3GT activity. Other normal human tissues will also be examined to see whether low level expression of these epitopes might be a general characteristic of human tissues. This may have important relevance with regard to anti-Gal mediated autoimmunity.

While the presence of the anti-Gal antibody in humans is well documented, the clonal analysis of its production has not been studied. In order to understand how the anti-Gal antibody, a polyclonal antibody, might be involved in the autoimmune disease process, it is of interest to study the specificity of anti-Gal secreting B lymphocyte clones. In addition, it will also be informative to study the relative abundance of circulating B lymphocytes which can secrete anti-Gal. The abundance of anti-Gal secreting cells will be compared with the abundance of cells secreting other natural antibodies in order to gain a greater understanding of the mechanisms of natural antibody production. These studies will provide a baseline for the future comparison of anti-Gal production

in normal versus diseased individuals and any changes in the distribution of anti-Gal secreting clones in these diseases.

Through these studies, we expect to lay a foundation for the understanding of the *in vivo* synthesis of α-Gal epitopes in man and other mammals and of the potential for anti-Gal mediated autoimmunity.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1). *Reagents. Bandeiraea simplicifolia* I-Sepharose (BS-Sepharose), *Bandeiraea simplicifolia* I lectin B_4 subunit (BS-lectin), biotinylated peanut agglutinin (PNA), fluorescein isothiocyanate-avidin (FITC)-avidin and the ABC reagent for the peroxidase reaction were purchased from Vector Laboratories, (Burlingame, CA) ^{125}I-streptavidin (specific activity 30μCi/μg) was purchased from Amersham Corp. (Chicago, IL). Uridine diphosphate-[^{3}H]galactose (UDP-[^{3}H]Gal, specific activity 20 Ci/mmol) was also purchased from Amersham Corp. Long-chain N-hydroxysuccinimidobiotin (NHS-d-biotin) was purchased from Sigma. Synsorb (silica) beads with linked synthetic oligosaccharides Galα1→3Galβ1→4GlcNAc-R and Galβ1→4GlcNAc-R were obtained from Chembiomed (Edmonton, Alberta, Canada). Bovine serum albumin, BSA, used in these studies was crystalline (Sigma Chemical Co., St. Louis, MO). Unless otherwise specified, all other reagents were purchased from Sigma.

2). *Glycoproteins.* Fibrinogen from various species and bovine fetuin were purchased from Sigma Chemical Co. (St. Louis, MO). Unless otherwise specified, all other glycoproteins were purchased from Sigma.

3). *Cell Lines.* The marmoset lymphoblast cell line, B 95.8, was obtained from the American Type Culture Collection (Rockville, MD). All other cell lines were obtained from the University of California, San Francisco Cell Culture Facility. Unless otherwise indicated, suspension cell lines were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 100U/ml penicillin/streptomycin, 2mM glutamine and 10% fetal calf serum. Adherent cell lines were maintained in Dulbecco's Minimal Essential Medium (DME; Gibco) with the same supplements. All cell lines were incubated under 5% CO_2 conditions at 37°C.

4). *Mammalian Tissues.* Frozen thyroid glands and sera from various mammalian species were obtained form Pel-Freez Biologicals (Rogers, AK) and primate thyroid glands were obtained from the Primate Center, University of California, Davis. At least two specimens from each species were analyzed. Nine normal human thyroid tissue specimens were received within one hour after death from the autopsy service at the University of Liège Hospital (Liège, Belgium). The specimens were immediately stored in liquid nitrogen, and the normal characteristics of the thyroid tissue were confirmed by histologic examination. Thyroid tissues were also received from eleven patients with Graves' disease and from four patients with nontoxic, nonprogressive goiter, all of which underwent thyroidectomy as part of their therapy (Liège, Belgium). The diagnosis of Graves' disease and of nontoxic goiter (performed by Dr. Roger Winand) was based on clinical history and examination, the results of standard diagnostic tests (i.e., high T_3, high T_4, flat TRH test, and increase in radioiodine uptake by the thyroid gland).

Diffused fixation of radioactive iodine by the thyroid glands in scintigraphy, the presence of thyrotropin-binding inhibiting immunoglobulins (TBII), and the presence of thyroid-stimulating immunoglobulins (TSI) indicated the occurrence of Graves' disease. Upon receipt, the specimens were stored in -70°C until they were subjected to analysis. Storage under these conditions was found, in separate assays, to preserve both α-galactosyl epitope expression and α1,3GT activity. Frozen thyroid glands from cow were purchased from Pel–Freeze (Rogers, AR). Human "buffy coats", used for the isolation of mononuclear cells and B lymphocytes, were obtained from the Irwin Memorial Blood Bank (San Francisco, CA).

B. Isolation and Biotinylation of Anti-Gal

Isolation of anti-Gal by affinity chromatography has been described previously (34). Briefly, 100ml batches of pooled, heat-inactivated AB plasma were loaded on a column containing 10ml of the synthetic Galα1→3Galβ1→4GlcNAc residues linked to Synsorb beads (Synsorb 115). After extensive washing with phosphate buffered saline (PBS), antibodies were eluted with cold glycine hydrochloride buffer, pH 2.6, and immediately neutralized with 0.1N NaOH. The eluate was dialyzed against PBS, pH 7.4, and diluted to a concentration of 100μg/ml. N-Hydroxy-succinimidobiotin (NHS-D-biotin, Sigma Chemical Co.) dissolved in dimethyl sulfoxide was added to the anti-Gal preparation at a final concentration of 0.2mg/ml. The mixture was incubated for 2h at room temperature and subsequently overnight at 4°C. After dialysis, to remove free

biotin, the biotinylated anti-Gal was rechromatographed on a Galα1→3Galβ1→4GlcNAc-R Synsorb column. The column was washed extensively with PBS and the bound anti-Gal was eluted by incubation with 0.2M methyl α-galactoside for 4h at 37°C. The free carbohydrate was removed from the eluted anti-Gal by four cycles of dialysis against a large volume (4L) of PBS. The antibody was then diluted to a concentration of 100μg/ml and stored in aliquots at -70°C. While the anti-Gal was substantially biotinylated, the extent of biotinylation could not be determined; . As seen in Figure 4, when the Galα1-3Galβ1-4GlcNAc structure is present on the solid-phase antigen, the binding results in a plateau of 40,000 cpm. The same concentration yielded only background counts with asialofetuin (Figure 1), indicating that this antibody is properly biotinylated and that it does not bind to the well-characterized carbohydrate residues of asialofetuin (11).

C. Preparation of Gal-13 Ascetic Fluid

Balb/c mice were injected intraperitoneally with 10^7 Gal-13 hybridoma cells (48) in 0.5ml Pristine (Sigma). After seven days, ascitic fluid was removed from peritoneal cavities and stored at -70°C. Titers of Gal-13 in ascitic fluid preparations by the direct agglutination of rabbit erythrocytes were 1/1000 to 1/2000.

D. Preparation of Glycoproteins.

Human and mammalian thyroglobulins were purified from thyroid glands by gel filtration (6). Murine EHS cell laminin and human laminin were kindly provided by Dr. J. Avila (University of Sao Paulo, Brazil). Desialation of fetuin was performed by incubation with 0.05 N H_2SO_4 for 2h at 80°C (75). IgG preparations from different species were obtained by protein-A Sepharose chromatography of sera, elution of the bound IgG with glycine hydrochloride buffer, pH 2.6, and subsequent neutralization and dialysis of the eluates. For digestion of glycoproteins with glycosidases, 200μg of each glycoprotein in 0.2ml was incubated for 14h with 0.4 unit of coffee bean α-galactosidase (Sigma) in sodium acetate buffer, pH 5.0. Glycoproteins were also treated with 30 units of bovine testis β-galactosidase in PBS for 14h. Similar treatment was performed with glycoproteins bound to microtiter wells.

Bovine thyroglobulin was fractionated for enrichment of the molecules containing high numbers of α-Gal epitopes on a BS lectin-sepharose affinity column. The BS lectin interacts with α-Gal epitopes (81). The affinity of single α-Gal epitopes for the BS lectin is low because only one of the four subunits can interact with such epitopes and the affinity of individual subunits for these epitopes is low (82). Therefore, we postulated that chromatography of glycoproteins on BS-Sepharose followed by extensive washing would result in the subsequent isolation of glycoprotein molecules with increased numbers of α-Gal epitopes, as compared with the average number per molecule in the original preparation. Bovine thyroglobulin in solution was passed through a 5-ml BS-Sepharose column. After extensive washing with PBS, the bound molecules

were eluted with 10mM methyl α-galactoside (6, 25) and dialyzed against PBS to remove the free carbohydrate. The concentration of eluted glycoproteins was determined by absorbance at 280nm. A similar procedure was carried out with other glycoproteins. These glycoprotein preparations were used to coat microtiter wells in the RIA assay.

E. Anti-Gal Binding to Glycoproteins in Microtiter Wells

Glycoproteins (50μg/ml) in 0.5M carbonate buffer, pH 9.5, were incubated overnight in Falcon 3912 microtiter plates (Becton Dickinson, Oxnard, CA) at 4°C. Each well contained 50μl of the glycoprotein solution. The wells were then washed with PBS containing 0.05% Tween 20 and subsequently incubated with 2% bovine serum albumin (BSA) for 2h at 37°C in order to block nonspecific binding. The plates were then incubated with 2-fold serial dilutions of biotinylated anti-Gal for 1h at room temperature. At the end of the incubation, the plates were washed with Tween-PBS and incubated 1h with ^{125}I-strepavidin (10^5 cpm/50μl). After the plates were washed again with Tween-PBS, 100μl of 0.2N HCL was added to the wells to detach the bound antibodies and the ^{125}I-strepavidin. The amount of ^{125}I-strepavidin was measured in a γ counter.

F. Immunoblotting Assays

1) *Dot Blotting.* Glycoproteins were diluted into carbonate buffer, pH 9.5. Two-fold serial dilutions of glycoproteins were added to wells of a dot blotting apparatus (Bio Rad, Richmond, CA). Proteins were then attached to nitrocellulose blotting paper (Biotrace NT, Gelman Sci., Ann Arbor, MI) by gravity flow through. Blots were then blocked with 1% BSA in PBS for 1h. BSA was removed and 50μl of 5μg/ml biotinylated anti-Gal in 1% BSA was added to wells. After 1h, anti-Gal was removed by suction and blots were washed extensively with 0.05% Tween-20 in PBS. Vector peroxidase ABC reagent was prepared 30 min. in advance in 1%BSA PBS. This avidin reagent was added to the wells in 50μl aliquots and incubated for 1h. Wells were then washed under suction extensively with Tween-PBS. Peroxidase substrate, 4-chloro-1-naphthol, (Bio Rad) was dissolved at 1mg/ml in 50mM Tris with 17% methanol, pH 7.4. Immediately before the addition of the substrate solution to the wells, hydrogen peroxide was added to a final concentration of 0.002%. The reaction was terminated by washing wells under suction with distilled water. All blotting procedures were carried out at room temperature.

2) *Western Blotting.* Fifty micrograms of various glycoproteins were separated by polyacrylamide (7%) gel electrophoresis (SDS-PAGE), and blotted onto nitrocellulose paper. After blocking of the nitrocellulose with 1% BSA and further blocking with a 10% solution of AB plasma depleted of anti-Gal (i.e., adsorption of plasma on Galα1→3Galβ1→4GlcNAc–Synsorb (Synsorb-115) and on washed rabbit red cells) for 1 h, the blotted proteins were subsequently covered with biotinylated anti-Gal (25 μg/ml) for 2 h at room temperature. Following washing with Tween–TBS, the bound

antibody was detected by a peroxidase reaction using the Vector ABC reagent and diaminobenzidine.

In order to demonstrate the specific interaction of anti-Gal with α–Gal epitopes on thyroid cell membrane glycoproteins, glycoprotein preparations were treated with α- or β-galactosidase prior to the Western blot analysis. Two hundred micrograms of membrane glycoprotein preparations were incubated for 14 h with 2.0 units of coffee bean α-galactosidase in 20 mM MES buffer, pH 6.0, or with 30 units of bovine testis β-galactosidase in PBS, pH 7.2.

G. Immunostaining of Thyroid Tissues with Anti-Gal

Frozen human and porcine thyroid tissues were embedded in OCT medium. Tissues were sectioned into 10μm slices using a cryostat. Sections were attached to glass slides which were coated with 0.05% polylysine. Sections were then pre-blocked with 1% crystalline BSA for 1 h at 4°C. Anti-Gal was isolated and biotinylated as described in Section B. Biotinylated anti-Gal at a 1:10 dilution was layered over the tissue sections for 1h, followed by extensive washing with PBS. Avidin-fluorescein (Vector) at a 1:300 dilution was then layered over sections for 1h, followed by extensive washing with PBS. Sections were photographed under fluorescence microscopy.

H. Quantification of α–Gal Epitopes by a Solid Phase Radioimmunoassay

1) *Quantification of α–Gal Epitopes on Various Glycoproteins.* Bovine thyroglobulin molecules, enriched for α–Gal epitopes, were isolated on BS-Sepharose from a commercial preparation of bovine thyroglobulin (Sigma) as described. This procedure results in the isolation of approximately 10% of the bovine thyroglobulin, which binds with high affinity to BS lectin and is designated BS+ thyroglobulin. This isolated thyroglobulin fraction, which provided the solid-phase α-Gal antigen, was diluted to a concentration of 50μg/ml in carbonate buffer, pH 9.5, and used to coat microtiter wells. In preliminary experiments, measuring the binding of biotinylated anti-Gal to BS+ thyroglobulin, an antibody concentration of 1μg/ml was found to be a suitable concentration for performing a highly sensitive solid-phase radioimmunoassay (RIA). For quantification of the number of α–Gal epitopes per molecule, each type of glycoprotein was incubated at different concentrations with biotinylated anti-Gal (1μg/ml) for 20h at 4°C. Subsequently, a 50μl aliquot of the anti-Gal-glycoprotein mixture was placed in the microtiter wells, which had been precoated with BS+ bovine thyroglobulin. Under these conditions, glycoproteins lacking the α–Gal epitopes did not inhibit anti-Gal binding to BS+ thyroglobulin coating the wells. However, glycoproteins with the α–Gal epitopes neutralized varying proportions of the biotinylated anti-Gal molecules, decreasing binding of anti-Gal to BS+ thyroglobulin. The degree of inhibition of anti-Gal binding to BS+ thyroglobulin was

assessed by the decrease in the subsequent binding of ^{125}I-streptavidin. To generate a standard curve for comparison, we used the data of Spiro and Bhoyroo (6), who found an average of 11 α–Gal epitopes per bovine thyroglobulin molecule. Thus, the concentration of this glycoprotein inhibiting 50% of anti-Gal binding (25nM) was used as a standard value for assessing the number of such residues on a variety of glycoproteins (see Figure 3 and Table II).

2). *Quantification of α–Gal epitopes on Thyroid Cell Membranes.* The radioimmunoassay (RIA) used for measuring α-galactosyl epitope expression on thyroid cell membranes is a slight modification of the RIA used for quantification of this epitope on soluble proteins, as was described in section 1. Briefly, the antibody used for the RIA was biotinylated anti-Gal at a concentration of 1 μg/ml, and the solid-phase antigen coating the RIA plates was bovine thyroglobulin enriched for α-galactosyl epitopes by affinity chromatography on BS–Sepharose. The RIA wells were coated with bovine thyroglobulin (20 μg/ml) in carbonate buffer, pH 9.5. When membranes from a variety of mammals were assayed, the 12,000 X g membrane pellets were first solubilized with 1% Triton-X 100 and then resuspended with biotinylated anti-Gal. When human thyroid membranes were examined, they were first fixed, as described, and then packed thyroid membranes were resuspended with 100-μl aliquots of anti-Gal. The mixture was incubated for 20 h at 4°C in microfuge tubes which were continuously rotated. Subsequently, the tubes were spun and the supernatants (50 μl) containing unbound anti-Gal were placed in the RIA wells in which the biotinylated anti-Gal bound to the solid-phase thyroglobulin.

After 90 min incubation, the plates were washed and the anti-Gal bound to the solid-phase α-galactosyl epitopes on bovine thyroglobulin was identified with ^{125}I-streptavidin. Membranes expressing α-galactosyl epitopes bound varying proportions of the biotinylated anti-Gal molecules, thus decreasing subsequent anti-Gal binding to the solid-phase α-galactosyl epitopes. Membranes lacking α-galactosyl epitopes displayed no inhibitory effect in this assay. It should be stressed that the studies of human tissues described in chapter VI were performed with fixed membranes rather than with solubilized membrane proteins, since the concentration of α-galactosyl epitopes on thyroid membranes was too low to permit their detection in a soluble form. The data were expressed as percent inhibition of anti-Gal binding at the various fixed packed membrane volumes.

3). *Quantification of α–Gal Epitopes on Cell Lines.* The method of quantification of α–Gal epitopes was essentially the same for cell lines as the described method for tissue membranes. Adherent cells were detached from culture flasks by washing once with calcium/magnesium-free PBS with 0.04% EDTA added, followed by incubation at room temperature with the same solution until cells could be detached by gentle agitation. Cells growing in suspension were washed three times with PBS. Cells were pelleted, resuspended in in PBS and glutaraldehyde was added to a final concentration of 0.2%. After a 1h incubation, cells were pelleted and resuspended in 0.2M glycine, pH 7.0, for another hour to block any unreacted glutaraldehyde. Cells were pelleted and resuspended in 5% blood type AB plasma depleted of anti-Gal, containing 0.05% sodium azide.

Plasma was depleted of anti-Gal by passage through a Synsorb 115 column, followed by adsorbtion with rabbit rbc. Fixed cells were stored at 4°C. Just before beginning the RIA, cells were pelleted and plasma was removed. Cells were aliquotted into four two-fold dilutions, beginning with 100µl of packed cells. Following the assay, the number of cells in each aliquot was determined by haemocytometer counting. To each cell pellet 100µl of an appropriate dilution of biotinylated anti-Gal was added. Cells were resuspended and mixed end-over-end overnight at 4°C. Cells were then pelleted and the supernatant was removed for the described RIA assay.

I. Rosetting Assay with BS-Lectin

This assay was performed as a modification of the procedure used by Galili et al. (34) for the measurement of the endpoint concentration of BS-lectin binding to various cells. This assay was utilized as a preliminary screening method because it is a rapid means of assessing the expression of α-Gal epitopes on cells. Since BS-lectin recognizes both α-Gal epitopes and the blood group B structure, the positive rosetting assays were always followed by the anti-Gal based RIA, which is highly specific for α-Gal epitopes. Only the RIA can be used practically for the studying of these epitopes on fixed membranes.

Adherent cells were washed once and released from culture plates with PBS, Ca^{2+} and Mg^{2+} free, plus 0.04% EDTA. Cells growing in suspension were were washed three times with PBS plus Ca^{2+}. Cells were then washed once with DME medium at 4°C. Cells

numbers were adjusted to 200,000 to 400,000 per 12x75mm polypropylene culture tube, pelleted and chilled to 0°C. Cells were resuspended in various concentrations of BS-lectin in DME medium and kept in ice. After 30 min, cells were washed three times with 4°C DME medium and pelleted. Freshly washed rabbit erythrocytes were suspended in DME medium at a concentration of 10% vol/vol. 100µl of the erythrocyte suspension was added to each culture tube with gentle mixing. Cells were pelleted at 4°C and allowed to remain pelleted for 30 min before resuspending them in 1ml PBS. The percentage of culture cells forming rosettes was determined by haemocytometer counting.

J. Microsome and Membrane Preparations

Thyroid specimens were homogenized in hypotonic Tris-buffered saline (TBS) (10 mM NaCl, 20 mM Tris, pH 7.4) containing 2 mM phenylmethylsulfonylfluoride (PMSF) with a Tekmar® homogenizer. The homogenized mixtures were fractionated according to Im et al. (83). The homogenates were spun at 1000 × g for 10 min, and the pellets containing unbroken cells and nuclei were removed. The supernatants were spun for 20 min at 12,000 × g and the resulting pellets contained the cell membranes. The supernatants were then spun at 40,000 × g for 30 min and the resulting pellets contained the microsomal fractions. The cell membrane fractions were washed twice in TBS to remove thyroglobulin, and resuspended in TBS with 1% Triton X-100. The microsomes were resuspended in TBS. Protein concentration in each fraction was determined by the method of Lowry et al. (84).

K. Isolation of the Bovine α1,3GT

The following description of the isolation of the bovine α1,3GT was performed as a modification of the method of Blanken and Van den Eijnden (58). α1,3GT was prepared by isolating microsomes from 200 g of bovine thymus. All steps involved in the purification of α1,3GT were carried out at 4°C. Microsomes were solubilized 4h with constant stirring in 20mM HEPES including 1mM PMSF, 1mM DTT, 20% glycerol and 1% NP-40, pH 7.0. The mixture was centrifuged at 20,000 × g for 90 min. Solid $MnCl_2$ was added to the supernatant to a final concentration of 5mM. The solution was stirred for 20 min and then the resulting precipitate was removed from the supernatant by centrifugation at 12,000 × g for 30 min. The supernatant was then passed through a 10ml GlcNAc-Synsorb column to remove proteins which have a tendency to bind non-specifically to Synsorb, as well as to remove β-galactosyltransferase activity. The effluent from this column was then passed through a 10ml Galβ1→4GlcNAc–Synsorb (LacNAc-Synsorb) column to bind α1,3GT activity. After washing the LacNAc-Synsorb column with 100ml of solubilizing buffer, the bound α1,3GT activity was eluted with 100mM n-Octyl glucoside in 20mM HEPES/glycerol, pH 3.0. The eluate was immediately neutralized with 0.2M carbonate buffer, pH 9.7. It was important to neutralize the eluate with carbonate since neutralization with NaOH resulted in the irreversible inactivation of α1,3GT activity. This may be the result of the formation of $Mn(OH)_2$, an insoluble salt. It was confirmed in these studies that removal of

Mn^{2+} from the enzyme solution results in an inactivation of enzymatic activity. α1,3GT enzymatic activity was determined by adding 50μl of the LacNAc-Synsorb column eluate to 100μl of the enzyme reaction buffer, which consisted of 100mM n-octylglucoside, 20mM MES, 5mM $MnCl_2$ and 2μCi [^{3}H]-UDP Gal, pH 6.0, plus 5mg LacNAc-Synsorb. The reaction was carried out for 90 min at 37°C. The amount of [^{3}H]-Gal incorporated onto the LacNAc-Synsorb beads was determined by scintillation counting. The α1,3GT containing eluates were concentrated in an Amicon concentrator (Amicon Inc., Danvers, MA.). Aliquots of concentrated eluate containing 1-2μg of protein, by 280nm absorbance, were subjected to SDS gel electrophoresis. Aliquots were diluted in SDS sample buffer, consisting of a final concentration of 1% SDS and 50mM DTT (dithiothreitol), in pH 8.1 Tris buffer. Samples were then electrophoresed in duplicate through a 7% polyacrylamide gel. The gel was divided in half. Half was stained with coumassie blue dye, while the other half was blotted onto Immobilon-P® paper (Millipore, Corp., Bedford, MA). An attempt was made to obtain a protein sequence from the major bands, blotted onto Immobilon-P paper, by the Protein Sequencing Facility at the Howard Hughes Medical Institute of UCSF.

L. Assessment of α1,3GT activity in microsomal fractions

The general methodology for studying α1,3GT activity involves the use of the acceptor Galβ1→4GlcNAc-R (LacNAc-R) and the radiolabeled substrate UDP-[^{3}H]Gal (or UDP-[^{14}C]Gal) in order to measure newly synthesized α-Gal epitopes (44, 53, 55, 56, 57, 58).

Measuring this enzyme in microsomal preparations from tissues that can be obtained in small amounts presents difficulties in accurate identification of α1,3GT activity. This is because microsomes contain a large variety of glycosyltransferases, including galactosyltransferases other than α1,3GT such as β1-4 galactosyltransferase, β1-3 galactosyltransferase (54), and α1-4 galactosyltransferase (87). In addition, the microsomal preparations are usually "contaminated" by lysosomes which contain β-galactosidase. This enzyme cleaves the terminal β-galactosyl residue of the Galβ1→4GlcNAc structure and generates an acceptor for β1-3 galactosyltransferase and β1-4 galactosyltransferase (86). The resulting synthesis of radiolabeled Galβ1→4GlcNAc-R or Galβ1→3GlcNAc-R structures may contribute to a false positive α1,3GT assay.

In view of these considerations, we concluded that a reliable assessment of α1,3GT activity should include two independent assays: a) an assay for measuring the rate of linkage of radiolabeled α-galactosyl residues to the lactosamine acceptor; and b) an assay for demonstration of the actual synthesis of Galα1→3Galβ1→4GlcNAc-R structures.

a) *Enzymatic activity measured by linkage of radiolabeled α-galactosyl residues* . The assay was carried out according to the method described by Elices and Goldstein (54), and measured the transfer of [^{3}H] Gal from the uridine diphosphate derivative to a synthetic LacNAc linked to Synsorb beads. Microsomal preparations were brought to a concentration of 10 mg/ml in MES buffer, pH 6.4, containing 20 mM $MnCl_2$, 1% Nonidet P-40, and the following

protease inhibitors: 10 μg/ml leupeptin, 50 μg/ml pepstatin A, and 50 μg/ml O-phenantrolene (Sigma). Aliquots of 50 μl of the microsomes were added to tubes containing 1 mg LacNAc–Synsorb, and 2 μCi of UDP-[^{3}H] Gal solution. The mixture was incubated for 4 h at 37°C and the beads were subsequently washed with TBS–0.05% Tween. The beads were then aliquoted into three equal volumes. One hundred microliter aliquots of α-galactosidase (1 unit/ml) in MES buffer and β-galactosidase (30 units/ml) in PBS buffer were added, respectively. The beads were incubated with these enzymes for an additional 20 h at 37°C. The radiolabeled galactose released by the enzymes into the supernatant was subsequently measured in a scintillation counter. The enzymatic activity was derived from the amount of [^{3}H] Gal released by α-galactosidase after subtraction of the [^{3}H] Gal in supernatants of beads incubated in H_2O. The possible contribution of β1-4 galactosyltransferase or β1-3 galactosyltransferase to the linkage of radiolabeled galactose to GlcNAc-R acceptors was assessed by measuring the amount of [^{3}H] Gal in the supernatant of beads treated with β-galactosidase. As argued above, the acceptor for these transferases may be generated as a result of lysosomal β-galactosidase cleaving terminal β-galactosyl residues on the LacNAc–Synsorb. The enzymatic activity was expressed as pmole [^{3}H] Gal per gram protein per hour.

b) *Demonstration of α-Gal epitope synthesis by α1,3GT.* This assay was based on the use of a solid-phase natural LacNAc-R acceptor on fixed rabbit red cells, UDP-Gal as substrate, and identification of the *de novo* synthesized α-Gal epitopes with a mouse

monoclonal antibody Gal-13, which is highly specific for these structures on glycolipids (48). Rabbit red cells express an abundance of α-Gal epitopes on glycolipids (22, 35) and readily bind the Gal-13 monoclonal antibody (48). Rabbit red cells at a concentration of 5% (vol/vol) in PBS were added in 50-μl aliquots to Falcon 3192 microtiter wells (Falcon, Oxnard, CA). After allowing the red cells to settle for 30 min, 25-μl aliquots of 1% glutaraldehyde were added to each well, causing the fixation of the rabbit red cells as single-cell monolayers bound to the bottom of the wells (89). After 15 min incubation, the glutaraldehyde and unbound red cells were removed by repeated washing with distilled water. The glutaraldehyde molecules bound to red cells and plastic were then neutralized by incubation of plates with 0.15 M glycine, pH 7, for 2 h. Subsequently, the glycine solution was removed, and monolayers of rabbit red cells were incubated overnight with 50-μl aliquots of α-galactosidase (1.0 unit/ml) in MES buffer, pH 6.0, at 37°C. This enzymatic treatment removes the terminal α-galactosyl unit from the α-Gal epitope on the rabbit red cells, exposing the penultimate Galβ1→4GlcNAc-R core structures, which subsequently serves as an acceptor for α1,3GT. After removal of α-galactosidase, the plates were washed with TBS. Prior to the enzyme assay, the microsomal preparations were brought to a concentration of 10 mg/ml in MES buffer, pH 6.4, containing 7.5 mM $MnCl_2$; and protease inhibitors as described above. These suspensions were extruded through a 28-gauge syringe to break microsomes. No detergent was added in order to prevent subsequent solubilization of glycolipids from the fixed rabbit red cells. Aliquots of 50 μl of the microsomes and

UDP-Gal at a concentration of 1 mM were added to each well containing the α-galactosidase-treated rabbit red cell monolayer. The plates were incubated for 20 h at 37°C in a humidified atmosphere and subsequently washed five times with Tween–TBS. Aliquots of 50 µl of Gal-13 antibody (ascitic fluid diluted 1:200 in 1% BSA–TBS) were added to the wells and incubated for 90 min at room temperature. The monoclonal antibody bound specifically to the *de novo* synthesized α-Gal epitopes. After removal of Gal-13 and washing five times with Tween–PBS, biotinylated horse anti-mouse IgG (Vector Labs) diluted 1:300 in 1% BSA–TBS was added, and the plates incubated for 60 min at room temperature. Both antibody preparations were preadsorbed on LacNAc-R Synsorb to remove natural anti-i/I antibodies. Subsequent to removal of the secondary antibody and washing of the plates, 50 µl of ^{125}I-streptavidin with a total of 10^5 cpm was added to each plate and incubated with the monolayer for 60 min. The unbound ^{125}I-streptavidin was then removed. The plates were washed, and the ^{125}I-streptavidin bound to the antibody on the monolayers was eluted with 100 µl 0.2 M HCl. The radioactivity in the eluates was measured in a gamma counter. Heat-inactivated microsomal preparations (90°C for 5 min) were used as controls. Enzymatic activity was expressed as cpm after subtraction of counts in the control wells. The assays were performed in triplicate.

M. Selection of Human B Cell Lines Expressing α-Gal Epitopes

1). *Transformation of Human B cells with Epstein-Barr Virus (EBV).* To remove red cells from human buffy coats, they were layered on Ficoll/Hypaque (1.077 g/ml) and spun for 20 min. at 1000 rpm in a Beckman model TJ-6 centrifuge. White blood cells remaining at the interface were removed and washed once with RPMI 1640. Sheep red cells, pre-treated with 0.14M aminoethylisothiocyanate (AET) for 20 min.(88), were mixed with the fractionated white blood cells and centrifuged for 2 min. at 1000 rpm. The pellet was incubated for 30 to 60 min. in ice, gently resuspended in RPMI 1640, layered over Ficoll/Hypaque and spun as before. Utilizing this procedure, T lymphocytes can be separated from B lymphocytes by the ability of T lymphocytes to form E-rosettes with treated sheep red cells. The B lymphocytes remain at the top of the interface, while the rosetted T lymphocytes are pelleted. It is necessary to remove T cells before transformation of B lymphocytes with EBV. Otherwise, the natural anti-EBV antigen killing activity of T lymphocytes will destroy the transformed B lymphocytes.

The lymphocytes, depleted of T lymphocytes, were then incubated overnight with a 1:1 dilution of 0.45μm filtered EBV containing supernatant of actively growing B95.8 transformed marmoset cells in the standard RPMI 1640 medium. The following day the medium was removed and replaced with fresh supplemented RPMI medium. After 9 to 14 days, proliferating colonies of cells could be clearly observed.

2). *FACS Selection of Cells with Cell Surface α-Gal Epitopes..* After 3 to 4 weeks, EBV transformed B cells formed actively growing

cell lines. At this stage cells were removed from flasks, pelleted, and resuspended in PBS. High affinity biotinylated anti-Gal was added to cell pellets in a 1:10 dilution. Cells were incubated at 0°C for 1h with the antibody. After washing the cells three times with ice cold PBS, avidin-fluorescein at a 1:300 dilution (Vector Labs, Burlingame, CA) in PBS was added at 0°C for 1h. After washing cells three times, propidium iodide was added at a concentration of 1-2μg/ml. Cells were then subjected to sorting by fluorescence activated cell sorting (FACS).

N. Transfection of Hela Cells with the mouse α1-3 Galactosyltransferase Gene

cDNA encoding the mouse α1,3GT gene (59) was obtained from Dr. John Lowe (University of Michigan). The α1,3GT gene was packaged in the pCDM7 expression vector (59) (kindly provided by Dr. Lowe). pCDM7 containing the bacterial neomycin phosphotransferase gene (conveying resistance to the neomycin analogue G418) was also obtained from Dr. Lowe. Both of these constructs were used as expression vectors and were stored in sterile water at 4°C. Co-transfections of HeLa cells, a type O human cervical carcinoma line (81), were accomplished by a calcium phosphate precipitation method, as follows. HeLa cells used for transfections were approximately 50% confluent in 60mm culture dishes. To form the calcium phosphate precipitate, plasmids containing the α1,3GT gene and the neomycin resistance gene were added to polypropylene tubes at 10μg and 1μg, respectively. In this experiment, plates were transfected in duplicate. Therefore, 20μg of plasmid was added to

0.5ml of 1/10 TE buffer (1mM Tris, 0.01mM EDTA, pH 7.0). 50µl of 2.5M $CaCl_2$ was then added and the tube was briefly vortexed. 0.5ml of HEPES buffered saline (280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, pH 7.08-7.15) was added dropwise to the plasmid containing tube with constant vortexing. The precipitate was allowed to form for 20 min. 0.5ml of precipitate was added per plate of HeLa cells with gentle swirling. HeLa cells were incubated in DME, supplemented as previously described for 4h in a tissue culture incubator. Medium was removed and cells were shocked with sterile 20% glycerol in PBS on a plate by plate basis. Glycerol was immediately removed and replaced by culture medium. Cells were washed 4 times to remove precipitate which was not bound to cells. Cells were then cultured in supplemented DME medium for 48h. Medium was removed and replaced with medium plus 1mg/ml G418 (Gibco, Grand Island, NY). After 3 days, the concentration of G418 was raised to 1.5mg/ml. HeLa cells were maintained in this medium for one month before selection of co-transfected cells for α-Gal epitope.expression. Larsen et al. (59) used BS-lectin to isolate cells expressing α-Gal epitopes following transfection with the α1,3GT cDNA. Therefore, selection of the transfected HeLa cells was performed by FACS, utilizing BS-lectin binding as a means of detecting α-Gal epitopes. Hela cells were incubated with biotinylated BS-lectin for 1h at 0°C followed by washing with DME medium and then incubation with FITC-Avidin (10µg/ml) for 1h. Cells were washed and suspended at a density of approximately 10^6/ml in DME medium plus 2µg/ml propidium iodide (dead cells will take up the propidium iodide). The cells were then subjected to FACS at the

brightest 1% of the HeLa transfectants were selected. Cells were expanded and subjected to another round of selection, using FACS. The final analysis was performed by using biotinylated anti-Gal (50μg/ml) as a probe for α-Gal structures.

G418 resistant HeLa cell co-transfectants were screened after the transfection for expression of cell surface α-Gal epitopes by utilizing the rapid BS-lectin rosetting technique. Cells were incubated in ice with 25μg/ml BS-lectin. Cells were washed 3 times with ice cold PBS. Freshly washed rabbit RBC were then added to transfectants, cells were mixed and pelleted at 4°C. The percentage of cells forming rosettes with rabbit RBC, cells expressing α-Gal epitopes, in cells co-transfected with the α1,3GT and neomycin resistance genes was compared with those transfected with the neomycin resistance gene alone by haemocytometer counting.

O. Antibody Dependent Cellular Cytotoxicity Assay

Adherent cell lines were plated into 24 well plates 24h before performing the cytotoxicity assay at a density of 40,000 cells per well. The following day, medium was replaced with 1ml medium containing 50μg/ml anti-Gal or medium alone. Freshly isolated mononuclear cells from normal donors were added to wells in two-fold serial dilutions, beginning at $2x10^6$ cells per well. After 24h and 48h wells were washed and cells were removed by trypsinization. To determine the level of cytotoxicity, the number of viable cells remaining was compared with control wells containing no anti-Gal.

CHAPTER III

DISTRIBUTION OF α-GAL EPITOPES ON SECRETED MAMMALIAN GLYCOPROTEINS AS MEASURED BY A SENSITIVE SOLID PHASE RADIOIMMUNOASSAY

Introduction

The structures commonly occurring on the carbohydrate chains of N-glycosylated glycoproteins have been reviewed in the introduction to this dissertation. Perhaps the most striking phenomenon associated with one of these structures, the Galα1→3Galβ1→4GlcNAc-R structure (α-Gal epitope), is the fact that humans produce quantities of the corresponding anti-Gal antibody in amounts of approximately 1% of circulating IgG. Galili et al (34) were able to find an evolutionary pattern in the distribution of terminal α-Gal epitopes on nucleated cells and erythrocytes. Whereas these structures were expressed on cells of many mammalian species, prosimians and New World monkeys, they were not found on cells of Old World monkeys, apes or humans. These studies also suggested that the distribution of α-Gal epitopes on cells was a result of the activity of the α1→3 galactosyltransferase (α1,3GT). The α1,3GT was found to be active in microsomal fractions from cells of New World monkey or nonprimate mammalian origin. However, this enzymatic activity was greatly diminished in cells of humans, apes or Old World monkeys. This suggested that the α1,3GT activity has been

evolutionarily suppressed in these mammals. This conclusion was based only upon the expression of the enzyme in cells grown *in vitro*. As was mentioned in the introduction to this dissertation, it is well established that cell lines may have altered patterns of glycosylation, and presumably altered glycosyltransferase activities. Therefore, it was necessary to determine whether glycoproteins produced *in vivo* display the same evolutionary pattern of expression of terminal α-Gal epitopes. To address this issue the expression of these structures was studied on the secreted glycoproteins, thyroglobulin, fibrinogen, and IgG, which were obtained from various species. The presence of α-Gal epitopes on these glycoproteins was demonstrated by Western blotting analysis and a newly developed solid phase radioimmunoassay.

Results

The specific interaction of anti-Gal with α-Gal epitopes on N-glycosylated glycoproteins. Anti-Gal was previously shown to interact specifically with α-Gal epitopes on glycosphingolipids or with such synthetic oligosaccharides. It did not interact with terminal Galα1→4Gal epitopes (P_1 antigen), β-galactosyl epitopes, or with other carbohydrate epitopes both on glycosphingolipids or synthetic oligosaccharides (23, 72). To determine the capacity of anti-Gal to serve as a reagent for detection of α-Gal epitopes on N-glycosylated glycoproteins, we examined its interaction with secreted glycoproteins which have chemically defined carbohydrate epitopes (Table II). Bovine fetuin has three N-linked and three O-linked carbohydrate chains. Both types of oligosaccharides (structures 1

and 2 in Table II) have terminal sialic acid epitopes (77, 11, 13). The results in Figure 1 demonstrate that anti-Gal did not interact with any of these carbohydrate epitopes on fetuin when this glycoprotein was attached to microtiter wells. Removal of the terminal sialic acid from fetuin results in the exposure of penultimate Galβ1→4GlcNAc and Galβ1→3GalNAc epitopes, respectively (Table II, structures 3 and 4). Anti-Gal also did not interact with these epitopes, as indicated by the complete negative binding to asialofetuin (Fig. 1). As a positive control for binding of asialofetuin in the microtiter wells, we used the lectin, peanut agglutinin, which interacts specifically with Galβ1→3GalNAc epitopes (107). Such epitopes are exposed on the O-linked carbohydrate chains of fetuin subsequent to the removal of the terminal sialic acid epitope (Table II, structure 4), and readily interact with the peanut agglutinin (Fig. 1).

An N-glycosylated glycoprotein, in which part of the terminal carbohydrate epitopes were biochemically characterized as Galα1→3Galβ1→4GlcNAc, is bovine thyroglobulin (71, 7, 6). Moreover, Spiro and Bhoyroo (6) reported that bovine thyroglobulin has an average of eleven such epitopes per molecule, whereas no such structures are present on human thyroglobulin. In concurrence, we have found that anti-Gal bound to bovine thyroglobulin, but not to human thyroglobulin (Fig. 2). Removal of the terminal α-Gal epitopes from bovine thyroglobulin by α-galactosidase (Table II, structure 6) eliminated its capacity to bind anti-Gal. As expected, treatment of bovine thyroglobulin with β-galactosidase had no effect on anti-Gal binding (Fig. 2).

These data on the specific binding of anti-Gal to α-Gal epitopes on bovine thyroglobulin, as well as former observations on anti-Gal specificity (23, 72), implied that this antibody is a suitable reagent for detecting these epitopes on N-glycosylated glycoproteins.

Table II

Interaction of anti-Gal with chemically defined oligosaccharide epitopes on bovine glycoproteins with N-linked or O-linked carbohydrate epitopes

Glycoprotein	Oligosaccharide Structure	Anti-Gal Binding
1. Fetuin	SA - Galβ1→4GlcNAc-R	–
2. Fetuin	SA - Galβ1→3GalNAc-Ser (Thr)	–
3. Asialofetuin	Galβ1→4GlcNAc-R	–
4. Asialofetuin	Galβ1→3GalNAc-Ser (Thr)	–
5. Thyroglobulin	Galα1→3Galβ1→4GlcNAc-R	+
6. Thyroglobulin (α-galactosidase)	Galβ1→4GlcNAc-R	–

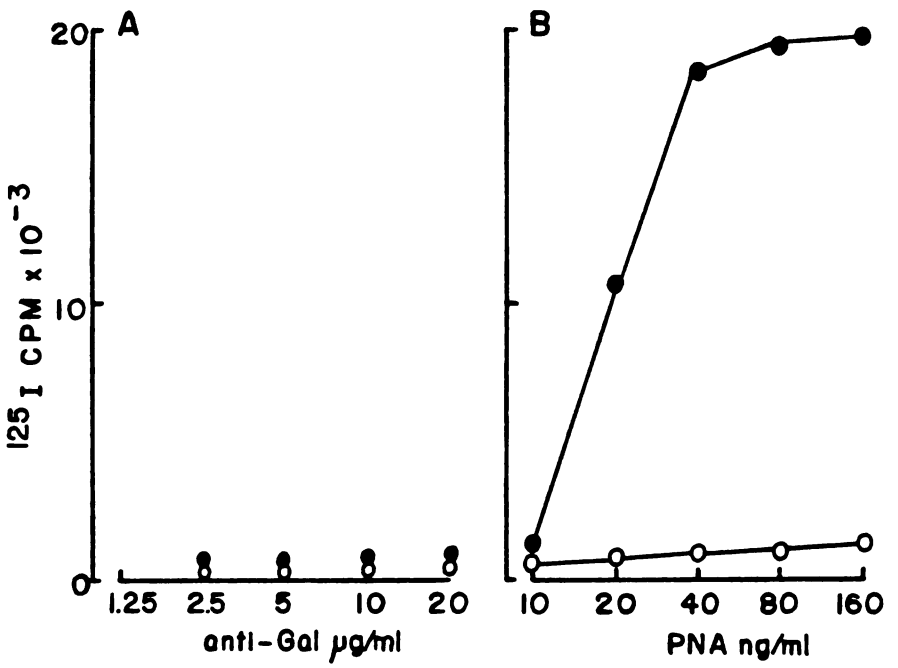


Figure 1: Binding of anti-Gal (*A*) and peanut agglutinin (PNA) (*B*) to bovine fetuin (○) and bovine asialofetuin (●) in microtiter wells.

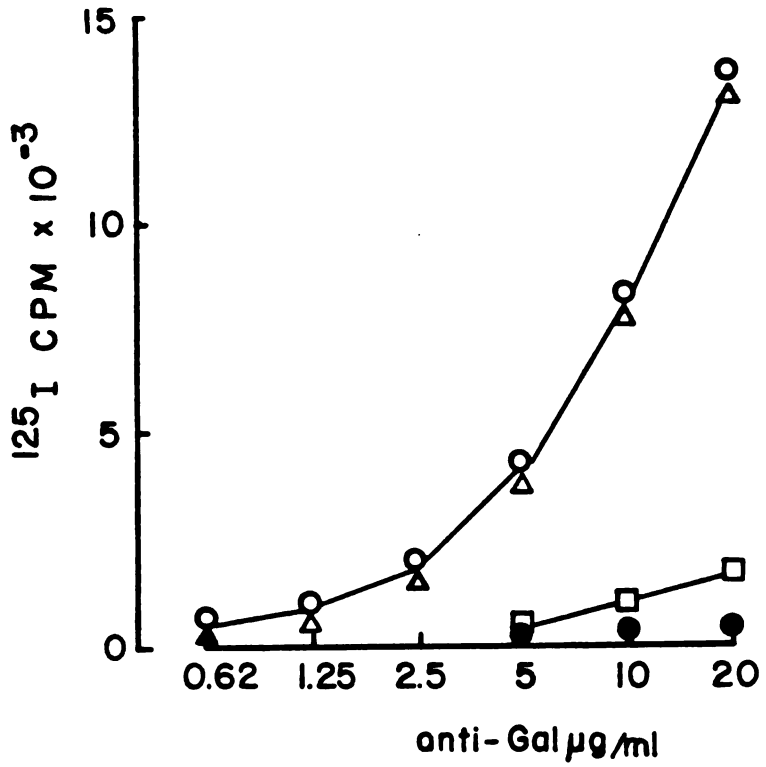


Figure 2: Binding of anti-Gal to thyroglobulin in microtiter wells. (○) Bovine thyroglobulin; (□) bovine thyroglobulin treated with α-galactosidase; (Δ) bovine thyroglobulin treated with β-galactosidase; (●) human thyroglobulin.

Quantification of α-Gal epitopes on thyroglobulin of various species. In order to determine the number of α-Gal epitopes on various secreted glycoproteins, we developed a modified solid-phase RIA in which bovine thyroglobulin enriched for α-Gal epitopes (BS^+ thyroglobulin) was used as a solid-phase antigen bound to microtiter wells. Inhibition of anti-Gal binding to the BS^+ bovine thyroglobulin subsequent to co-incubation of the antibody with various glycoproteins in solution was dependent on the presence of α-Gal epitopes on the glycoproteins tested.

Bovine thyroglobulin at a concentration of 25 nM yielded 50% inhibition of anti-Gal binding (Fig. 3, Table III). The bovine BS^+ thyroglobulin concentration causing 50% inhibition was only 9 nM (Fig. 3, Table II). By using Spiro and Bhoyroo's (6) data of an average of eleven α-Gal epitopes per bovine thyroglobulin molecule as a standard, the number of such epitopes on the BS^+ bovine thyroglobulin could be estimated to be thirty per molecule. The number of these epitopes on porcine thyroglobulin was estimated to be six per molecule (Table III). This is similar to the value reported by Spiro and Bhoyroo, who measured the release of terminal α-galactosyl epitopes by α-galactosidase. Furthermore, the inhibition curve with porcine thyroglobulin paralleled that of bovine thyroglobulin. Thus thyroglobulin concentration for a given inhibition was approximately double with porcine as with bovine thyroglobulin (not shown). The α-Gal epitope was also found to be expressed in varying amounts on thyroglobulin molecules of other mammals, including mouse, rat, hamster, rabbit, and dog (Table III).

When primate thyroglobulins were studied, this epitope was readily detected on squirrel monkey (a New World monkey) thyroglobulin in amounts comparable to those found in various nonprimate mammals. This epitope was not found, however, on rhesus monkey (an Old World monkey) thyroglobulin or on human thyroglobulin (Fig. 3, Table III), as indicated by the fact that thyroglobulin molecules from both species, even at the high concentration of 15 μM, had no inhibitory effect on the binding of anti-Gal to the solid-phase BS^+ bovine thyroglobulin.

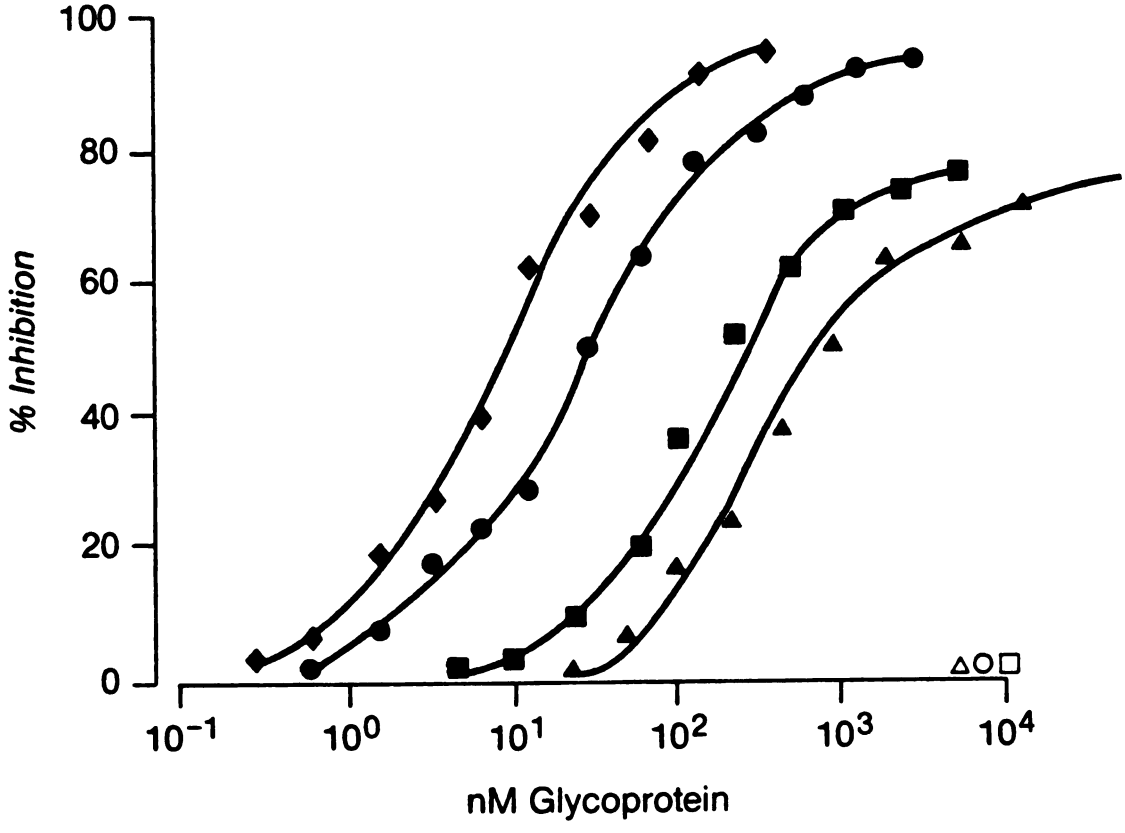


Figure 3: Solid-phase RIA based on inhibition of anti-Gal (1 μg/ml) binding to bovine BS^+ thyroglobulin, by preincubation of the antibody with various glycoproteins. (●) bovine thyroglobulin; (◆) BS^+ bovine thyroglobulin; (■) goat fibrinogen; (▲) mouse monoclonal IgG; (Δ) bovine IgG; (○) human thyroglobulin; (❑) human fibrinogen.

Table III

Quantification of α–Gal epitopes
on various glycoproteins as measured in solid-phase RIA

Glycoprotein[c]	Species	50% Inhibition [nM]	α–Gal epitopes : molecule (approximation)
Thyroglobulin	Bovine	25	11[a]
	BS$^+$ bovine[b]	9	30
	Mouse	800	0.25
	Rat	420	0.5
	Guinea Pig	450	0.5
	Hamster	1500	0.14
	Rabbit	130	2
	Pig	45	6
	Squirrel monkey (N.W. monkey)	300	1
	Rhesus monkey (O.W. monkey)	no inhibition at 15 μM	–[b]
	Human	no inhibition at 15 μM	–
Fibrinogen	Bovine	88	3
	Porcine	83	3
	Sheep	150	2
	Horse	4000	0.06
	Cat	41	7
	Goat	200	1
	Rabbit	3500	0.08
	Dog	860	0.3
	Baboon (O.W. monkey)	no inhibition at 15 μM	–
	Human	no inhibition at 15 μM	–

(continued on next page)

Table III
(cont'd.)

Glycoprotein	Species	50% Inhibition [nM]	α-Gal epitopes per molecule (approximation)
IgG	Mouse mAb	505	0.5
	Bovine	no inhibition at 17 μM	–
	Porcine	no inhibition at 17 μM	–
	Sheep	10,000	0.03
	Horse	no inhibition at 20 μM	–
	Rabbit	16,000	0.02
	Dog	8000	0.03
	Aotus (N.W. monkey)	33,000	0.01
	Squirrel monkey (N.W. monkey)	22,000	0.013

[a] This value was obtained from the study of Spiro and Bhoyroo (1984) and was used as a standard value for the RIA.

[b] – (minus) represents no measurable α-Gal epitopes.

[c] Glycoprotein preparations were pooled from two individuals or more, except for human thyroglobulin, which was isolated from two individuals assayed separately.

Studies on mammalian fibrinogens. Townsend et al. (10) have shown that human fibrinogen molecules have four N-linked carbohydrate chains with the terminal SA-Galβ1→4GlcNAc structure. The pattern of anti-Gal binding to fibrinogen molecules from various species suggested that the α-Gal epitope is present on bovine and porcine fibrinogen (Fig. 4). Affinity chromatography of bovine and porcine fibrinogen on BS–Sepharose resulted in the isolation of approximately 1% of the fibrinogen molecules with a high affinity for BS lectin. These fibrinogen molecules expressed more α–Gal epitopes than the average number, as seen in the increased interaction with anti-Gal (Fig. 4). This interaction could also be demonstrated in SDS–PAGE followed by Western blotting and immunostaining with anti-Gal (Fig. 5). No human fibrinogen could be recovered following chromatography on BS-Sepharose. Thus, the human fibrinogen used for these studies was the original preparation, obtained from Sigma. No anti-Gal binding was observed with human fibrinogen (Fig. 4, Fig. 5). By using the RIA, the average number of α–Gal epitopes per molecule of fibrinogen could be determined. The porcine and bovine fibrinogens were found to have approximately three α–Gal epitopes per molecule (Table III, Fig. 3). Assays with fibrinogen from other mammalian species indicated variable expression of the carbohydrate structure in numbers ranging from seven epitopes per molecule in cat fibrinogen, to an average of one epitope per seventeen molecules of horse fibrinogen (Table III). In accordance with the direct binding assay (Fig. 4), no interaction between anti-

Gal and human or baboon fibrinogen was observed in the RIA even at a concentration of 15 μM of the glycoprotein (Table III, Fig. 3).

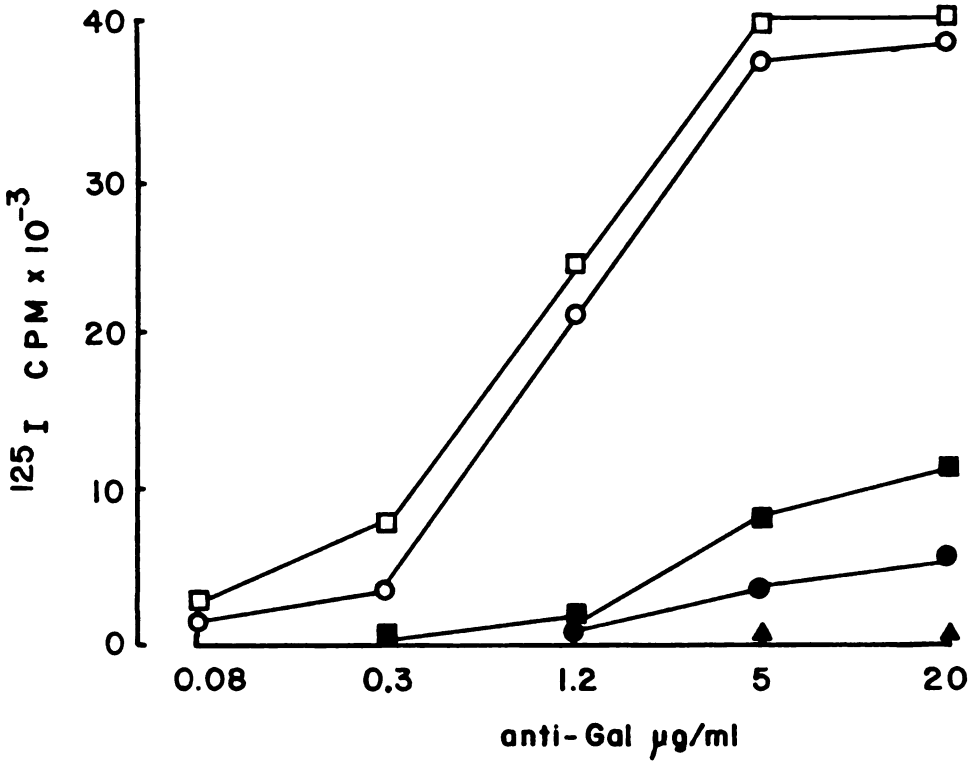


Figure 4: Binding of anti-Gal to bovine (■), porcine (●) and human (▲) fibrinogen in microtiter wells. Open symbols represent fibrinogens fractionated on BS–Sepharose.

Figure 5: Western blot and anti-Gal immunostaining of fibrinogens obtained from different species. The bovine and porcine fibrinogens were isolated on BS–Sepharose prior to the SDS–PAGE procedure.

KDa
200
116
66
45
31
Bovine Fibrinogen
Porcine Fibrinogen
Human Fibrinogen

α-Gal epitopes on IgG molecules. The IgG molecules of both mouse (76) and man (73) were reported to have two N-linked carbohydrate chains with terminal SA-Galβ1→4GlcNAc residues. In view of our recent studies (34) on the expression of α-Gal epitopes on mouse myeloma cells, and the activity of the enzyme α1→3 galactosyltransferase in these cells, we hypothesized that the secretory product, i.e., IgG molecules synthesized by mouse myeloma cells, may also have α-Gal epitopes. To study this possibility, we used the mouse monoclonal IgG antibody, designated Gal-13 (48), as a representative mouse monoclonal IgG. As seen in Figure 6, anti-Gal bound to the heavy chain of mouse IgG. This binding was not affected by treatment of the IgG molecules with β-galactosidase. However, pre-exposure to α-galactosidase completely eliminated the binding. As expected, anti-Gal did not bind to human IgG (Fig. 6).

The mouse monoclonal IgG antibody assayed by the RIA was found to have an average of 0.5 α-Gal epitopes per IgG molecule (Table III). No such epitopes were detected on bovine or porcine IgG. However, small numbers of α-Gal epitopes were detected on IgG molecules from sheep, rabbit, dog, and New World monkeys. Similar studies could not be performed with IgG from Old World monkeys, apes, or man, since these species have large amounts of anti-Gal (30) which would pre-bind to any autologous IgG expressing α-Gal epitopes. Thus, these structures would be undetectable in the RIA.

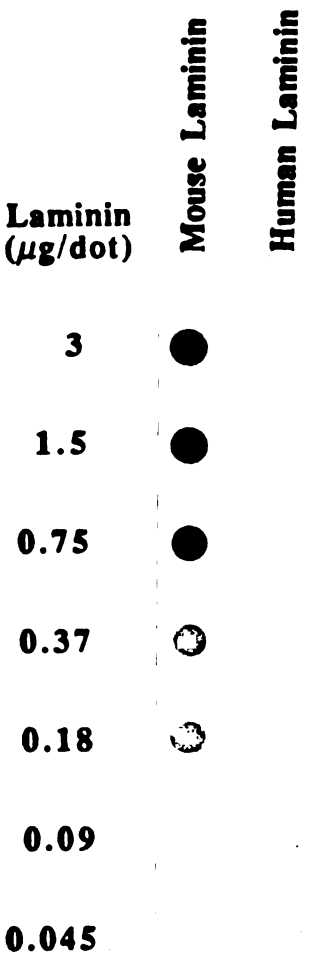
Figure 6: Western blotting of mouse and human IgG and subsequent immunostaining with anti-Gal. The mouse IgG was also pretreated with α- or β-galactosidase.

kDa
200
116
66
45
31
Mouse IgG
Mouse IgG
α-galactosidase
Mouse IgG
β-galactosidase
Human IgG

α-Gal Epitopes on Laminin.

Laminin is a prominent component of extracellular matrices. The N-linked carbohydrates of murine laminin have previously been shown to contain from 50 to 70 α-Gal structures per molecule (25, 9). Studies in the past suggested the absence of the α-Gal structure on human laminin (9). However, this study relied upon chemical methods, which require a greater amount of glycoprotein to detect the epitopes than methods of detection utilizing anti-Gal. Therefore, it was of interest to directly compare the ability of anti-Gal to interact with murine and human laminin. A dot blotting assay was utilized in order to illustrate the use of an alternate methodology for the rapid visual assessment of the presence of α-Gal epitopes on glycoproteins. Laminins were blotted in a dot-blot apparatus (Bio-Rad, Richmond, CA) in decreasing amounts and biotinylated anti-Gal was allowed to interact with the dots (Fig. 7). Whereas anti-Gal could visibly interact with murine laminin to a concentration of 0.09μg/dot, human laminin showed no interaction with anti-Gal. There was a weak staining of the human laminin at 3μg/dot. However, this is probably nonspecific staining due to a trapping of anti-Gal by the high concentration of bound laminin.

Figure 7. Binding of Biotinylated anti-Gal to mouse and human laminin in a dot-blot assay. Laminin was blotted as described in Materials and Methods.


Laminin (μg/dot)
Mouse Laminin
Human Laminin
3
1.5
0.75
0.37
0.18
0.09
0.045


Laminin (μg/dot)
Mouse Laminin
Human Laminin
3
1.5
0.75
0.37
0.18
0.09
0.045

Discussion

The use of antibodies, mostly of the monoclonal type, for identifying carbohydrate structures on cells and on isolated glycoconjugates, has increased in recent years (108). Although this approach is qualitative rather than quantitative, it does provide a sensitive assay for the detection of a unique carbohydrate structure when such a structure is a minor component on cells or within a heterogeneous population of oligosaccharides linked to a given protein.

The natural human antibody, anti-Gal, used in the present study for the identification of α-Gal epitopes, although not a monoclonal antibody, is highly specific for this carbohydrate structure on mammalian glycoproteins. Anti-Gal was shown previously to interact with melibiose (Galα1→6Glc) (16, 23). However, no Galα1→6Gal structures have been detected on mammalian glycoproteins.

The biochemical quantification of the number of α-Gal epitopes on bovine thyroglobulin (6) enabled us to use this molecule as a convenient standard for establishing the RIA for the quantification of this epitope. In addition to their finding of 11 epitopes of α-Gal epitopes on bovine thyroglobulin, Spiro and Bhoyroo (6) reported that pig thyroglobulin molecules have half as many terminal α-Gal epitopes as bovine thyroglobulin. In accordance, the RIA measurements indicated that pig thyroglobulin has six α-Gal epitopes, further demonstrating the accuracy of the RIA

quantification. The assay described in the present study may serve as a general method for assessing the number of a given carbohydrate epitope on various glycoproteins by using the appropriate antibody and corresponding solid-phase antigen.

The pattern of anti-Gal binding to thyroglobulin from various mammals suggested a distinct distribution of α-Gal epitopes among various species. These epitopes were detected on thyroglobulin molecules from many nonprimate mammals. It is interesting to note that, the number of α-Gal epitopes per molecule varied greatly in thyroglobulin of different species, whereas in all fibroblasts examined from various nonprimate mammals, there was a high number of cell membrane α-Gal epitopes (34). It was found to be abundant on bovine and porcine thyroglobulin, but scarce on thyroglobulin of mouse or rat. This is interesting since murine laminin, another glycoprotein studied, has an abundant number of α-Gal epitopes. The interaction of murine laminin with anti-Gal was clearly shown in the dot-blot assay. Therefore, one might speculate that a reason for this difference is due to the differential activity of the α1,3GT in the murine thyroid cells and the cells secreting laminin. The α-Gal epitope was demonstrated on New World monkey thyroglobulin, but not on Old World monkey and human thyroglobulin. This epitope was also not found in the dot-blot assay of human laminin with anti-Gal.

This pattern of distribution of α-Gal epitopes in mammals is apparent, not only with thyroglobulin and laminin, but also in other secreted glycoproteins which thus far have been assumed to have only SA-Galβ1→4GlcNAc-R structures, i.e., fibrinogen and IgG

molecules. The occurrence of the α-Gal structure on mouse monoclonal IgG antibodies has been recently confirmed by Krotkiewski et al. (74) who demonstrated this structure on the mouse monoclonal antibody, OKT3, by the use of ^{1}H-NMR and fast atom bombardment mass spectrometry. These findings suggest that the pattern of distribution of α-Gal epitopes observed on red cell and nucleated cell membrane glycoconjugates of various mammalian species (72, 33, 34) is also applicable to secreted N-glycosylated glycoproteins. It should be stressed that the data obtained in the radioimmunoassay are an approximation of the number of carbohydrate epitopes per molecule. It is conceivable that in suspension, if α-Gal epitopes are distant from each other on a glycoprotein molecule, the interaction of anti-Gal with this epitope would be in a 1:1 ratio, whereas if clustered, one anti-Gal IgG molecule would probably bind two epitopes. Nevertheless, this assay provides information on whether a given glycoprotein carries a large number, few, or no such epitopes.

The capacity of synthesizing the α-Gal epitopes on secreted glycoproteins seems to reflect a differential activity of the enzyme α1→3 galactosyltransferase in the Golgi apparatus. The activity of this enzyme was demonstrated in cells from mouse (34, 44, 55, 53), rabbit (56, 57), bovine (34, 58) and New World monkey (34) origin. Studies undertaken during this dissertation project indicate that, in addition to the synthesis of α-Gal epitopes on membrane glycoconjugates (30, 34), this enzyme is also responsible for the synthesis of these epitopes on different types of secreted glycoproteins in nonprimate mammals and New World monkeys. In

Chapter V, the enzymatic activity responsible for the synthesis of α-Gal epitopes will be analyzed, using the mammalian thyroid as a model tissue.

The most striking pattern to emerge from the present study is the absence of α-Gal epitopes on Old World monkey and human secreted glycoproteins. Since the enzymatic repertoire for synthesizing LacNAc acceptors on N-glycosylated glycoproteins is present and active in humans (1), the lack of α-Gal epitopes on human N-glycosylated glycoproteins seems to be the result of diminished activity of the α1,3GT. The cDNA of the α1,3GT has been cloned by Lowe et al. from a mouse cDNA library (59, 75) and by Joziasse et al. (60) from a bovine cDNA library. Southern blot analysis using these cDNA probes revealed homologous sequences in human DNA, suggesting that the α1,3GT gene has been evolutionarily conserved in humans (60, 62). The work of Galili et al. (34), suggested that, while the gene may be present in humans, it is largely inactive. However, recent studies suggest that human cells may aberrantly express α-Gal epitopes. In one such study, human vascular and placental tissue plasminogen activator were found to express α-Gal epitopes (28). Castronovo et al. (61) have also reported that human mammary carcinoma cell lines and the malignant cells from primary lesions of patients with mammary carcinoma readily bind both anti-Gal and BS lectin. These studies suggest that the α1,3GT gene, which appears to be suppressed in man, may undergo deregulation, resulting in the increased synthesis of α-Gal epitopes on human glycoconjugates. Deregulation of human α1,3GT *in vivo* may be of pathologic consequence, since it could

result in the initiation of an autoimmune process mediated by anti-Gal binding to the *de novo* synthesized α-Gal glycoconjugates (70). The sensitive RIA assay developed in this study was utilized, as presented in the following chapters, for the further study of α-Gal epitopes on human cells.

CHAPTER IV

Aberrant Expression of The α–Gal Epitope on Transformed Human Cell Lines

Introduction

α-Gal epitopes have been shown to be expressed in large numbers on cells of nonprimate mammals and cells of New World monkeys. Preliminary studies by Galili et al. suggested that human cells may not normally express these epitopes (34). However, studies by Castronovo et al. (61) of human mammary carcinoma cells suggested that such malignant cells aberrantly express the α-Gal epitopes. Studies have also suggested that humans may have the capacity for the expression of these epitopes *in vivo* in a cryptic form on erythrocytes (66). Therefore, it was of interest to determine the propensity of transformed human cells to express α-Gal epitopes. In this study, the highly sensitive RIA described in Chapter III was utilized for the detection of α-Gal epitopes on a variety of transformed human cell lines. The amount of anti-Gal binding to these cells was compared with the binding to mouse cell lines in order gain an idea of the relative numbers of these epitopes. As an additional confirmation of the interaction of anti-Gal with these human cells, the interaction of BS-lectin with the same cell lines was assessed. Furthermore, the ability of anti-Gal to mediate lysis of transformed human cells aberrantly expressing α-Gal epitopes was assessed by employing an antibody dependent (anti-Gal) cytotoxicity

assay (ADCC), using human blood lymphocytes as killer cells. As will be described, this study demonstrates that some transformed human cells can express cell surface α-Gal epitopes. The results of this study also provide support for the theory, proposed by Galili, (70) that aberrant expression of terminal α-Gal epitopes on human cells can result in an immune mediated destruction of such cells. As has been argued in this dissertation, the binding of anti-Gal to human cells could hypothetically represent an initiating factor in autoimmune diseases

Results

RIA for Quantification of α-Gal Epitopes on Cells. Studies which attempted to determine whether nucleated human cells have cell surface α-Gal epitopes were not able to detect such epitopes on several human cell lines (34). The techniques utilized in these early studies involved a measurement of the direct binding of radiolabeled BS-lectin or anti-Gal visualized by immunofluorescence (34). Both of these techniques have an insufficient sensitivity for the detection of low number of α-Gal epitopes on cells. BS-lectin is a tetramer and the individual subunits have a low affinity for α-Gal epitopes (84). Thus, if these epitopes are dispersed at a low density on human cells, the interaction with single BS-lectin subunits might not be sufficient to withstand the washing procedures of the assay. Detection of α-Gal epitopes by direct anti-Gal binding utilizing visual immunofluorescence can be subjective and a number of factors could limit the sensitivity of this method. Since the data from the study of Galili et al. (34) indicated that human cells would probably possess

fewer epitopes than nonprimate mammals, a more sensitive method for the detection of α-Gal epitopes was needed. Therefore, the RIA described in Chapter III was used for the detection of low numbers of α-Gal epitopes on human cells. Since this RIA measures the adsorbtion of anti-Gal to epitopes on the cell surface, it has the advantage of the high specificity of anti-Gal for α-Gal epitopes combined with little dependence upon the affinity of the interaction (there is no washing of cells, which may detach the bound IgG). The RIA is devoid of the subjective interpretation of weak immunofluorescence assays, as it relies upon a quantitative detection of biotinylated anti-Gal bound to antigen (BS^+ thyroglobulin) coated wells.

As has been mentioned in previous discussions, it is believed that the synthesis of α–Gal epitopes has been suppressed in human cells. Using the RIA, described above, a number of transformed human cell lines were assayed for the expression of these epitopes. As standards for the quantification of α–Gal epitopes, murine SP/2 mouse myeloma cells, previously found by Scatchard analysis with BS lectin to have at least 1.2×10^6 such epitopes per cell (34), and murine NIH 3T3 fibroblasts (43) were used. Figure 8 shows the results of the RIA, based on the inhibition of anti-Gal binding to wells coated with BS^+ thyroglobulin, with these murine cell lines. RIA inhibition curves of fixed murine SP/2 or NIH 3T3 cells indicated that these cells could adsorb 50% of anti-Gal binding activity at a density of $0.7\text{-}1.5\times10^4$ cells. The adsorbtion of anti-Gal binding activity was completely abolished by pretreatment of cells with α–galactosidase. When transformed human cell lines were assayed

with this RIA, several cell lines were found to express α–Gal epitopes. These included the lung carcinoma cell line NCI H69 (82) and the osteosarcoma cell line MG 63(80). The NCI H69 and MG 63 cells were examined in parallel with the murine cell lines in the RIA in order to assess the number of epitopes on these human cells relative to the number of epitopes on the murine cells. In this assay, the inhibition curves for both transformed human cell lines indicated a 50% inhibition of anti-Gal activity at around $8x10^6$ cells (Figure 8). Since there are roughly 500 times the number of anti-Gal binding sites per cell on SP/2 cells versus NCI H69 or MG 63, these human cell lines would be expected to possess at least 2,400 sites per cell. Pretreatment of fixed NCI H69 cells with α-galactosidase resulted in a complete inhibition of of their interaction with anti-Gal. This suggests that anti-Gal interacts with these human cells via a specific interaction with α-Gal epitopes. As a negative control for anti-Gal binding, CHO cells, which do not express α-Gal epitopes (111), were utilized. A human carcinoma cell line, H498, was not found to interact with anti-Gal in the RIA and is included in Figure 8 as a human cell line negative control. At 10^7 cells, a negligible inhibition of anti-Gal binding was observed with these control cell lines. Thus, Figure 8 shows the large difference in the number of α-Gal epitopes on murine cell lines compared with transformed human cell lines and the specificity of this interaction.

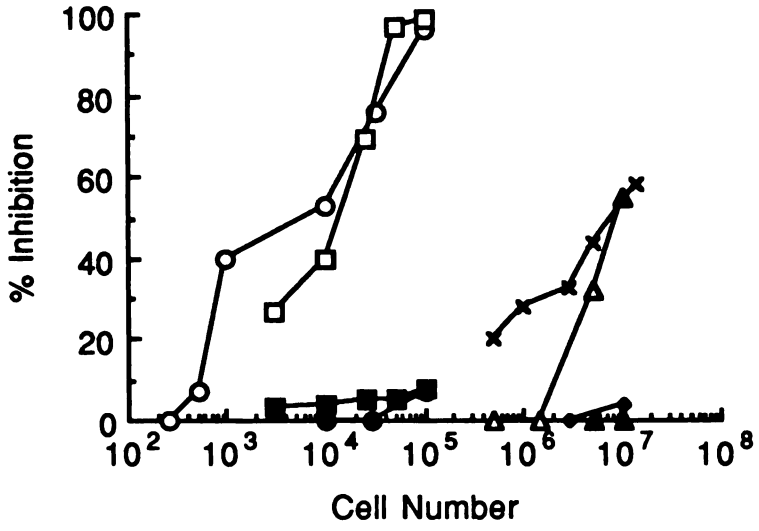


Figure 8. RIA based on the inhibition of anti-Gal binding BS^+ thyroglobulin by preincubation with cell lines. (□) murine SP/2 myeloma cells; (■) SP/2 cells pretreated with α-galactosidase; (○) murine NIH 3T3 fibroblasts; (●) NIH 3T3 cells pretreated with α-galactosidase; (Δ) human NCI H69 cells; (▲) NCI H69 cells pretreated with α-galactosidase; (×) human MG 63 cells; (◊) chinese hamster ovary cells (CHO) and human H498 cells. The CHO and H498 cells bound approximately the same amount of anti-Gal.

Figure 9 shows the results of RIA's with three biotinylated anti-Gal preparations from blood group AB plasma of different individuals. As shown, anti-Gal preparations 2 and 3 had approximately the same overall binding and adsorbtion characteristics. However, more binding to BS+ thyroglobulin coated wells was observed with preparation 1. While there are quantitative differences in the amount of anti-Gal bound to the wells, the number of cells at 50% inhibition of binding is the same in the three antibody preparations. The 50% inhibition cell number for NIH 3T3 cells is approximately 10^4 cells. For NCI H69 cells this inhibition of anti-Gal binding is observed at $3x10^6$ cells. This indicates that the binding characteristics of the three anti-Gal preparations of Figure 9 are in close agreement with the binding observed by the pooled anti-Gal, as seen in Figure 8. Therefore, the ability to perform the RIA with human α-Gal positive cells, with both of these types of anti-Gal preparations, indicates that the interaction with α-Gal epitopes on human cells is characteristic of anti-Gal from many and probably all individuals.

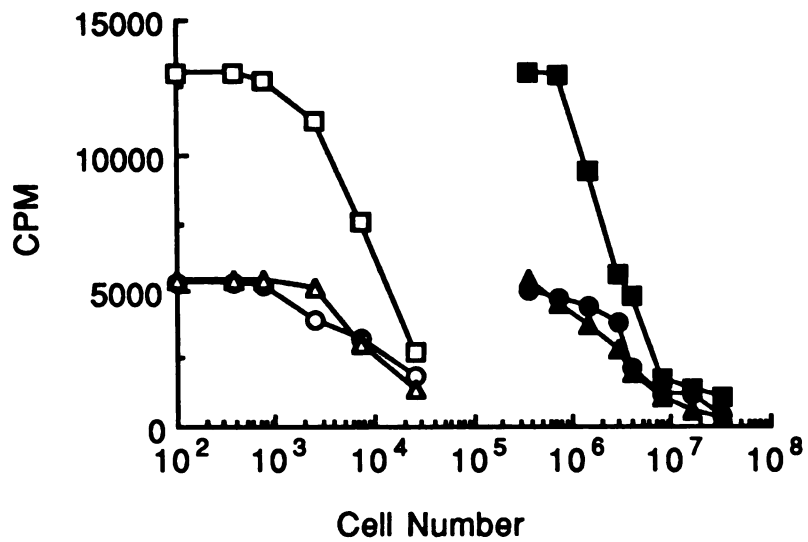


Figure 9. RIA based on the inhibition of binding of anti-Gal from three different individuals by human NCI H69 cells, filled in symbols and murine NIH 3T3 cells lines open symbols. Anti-gal was prepared as described in the Material and Methods section from blood group AB sera of three different individuals, number 1 (□); number 2 (○); and number 3 (Δ). Values are expressed in counts per minute ^{125}I, released from RIA plates.

The Ability of Anti-Gal to Promote Cell Mediated Cytolysis. The results of the experiments represented in Figures 8 and 9 suggest that α-Gal epitopes are present on the human cell lines NCI H69 and MG 63 in numbers ranging from 2500-10,000 per cell. Therefore, it was of interest to determine if the small number of anti-Gal binding sites per cell were sufficient to mediate antibody-dependent cell-mediated cytolysis (ADCC). ADCC occurs when antibodies bind to the cell surface of target cells, followed by killer T-cell activation, mediated by F_c receptor binding to the antibodies on the target cells. Cell lines were incubated in the presence of absence of anti-Gal at 20μg/ml under standard tissue culture conditions (see Material and Methods). As is seen in Figure 10, human mononuclear cells were added to cell lines at concentrations of $2x10^6$ to $0.25x10^6$. ADCC is expressed as % cytotoxicity at various mononuclear cell numbers per well (24 well plates). As Figure 10 indicates, anti-Gal can mediate ADCC with NCI H69 and MG 63 cells. The difference between the presence and absence of anti-Gal is most pronounced at mononuclear cell concentrations from $0.5x10^6$ to $1.0x10^6$ cells per well. At high mononuclear cell concentrations the elevated cytotoxicity may be due to natural killer cell activity, which is distinct from the ADCC reaction in that it is not mediated by antibodies. As controls, H498 and CHO (111) cells were subjected to ADCC assays under the same conditions as the NCI H69 or MG 63 cells. No cytotoxicity was observed with these cell lines, either in the presence or absence of added anti-Gal. The results, as seen in Figure 10 and summarized in Table IV, suggest that there are a sufficient number of α-Gal epitopes on some human cell lines to cause ADCC in the presence of anti-Gal.

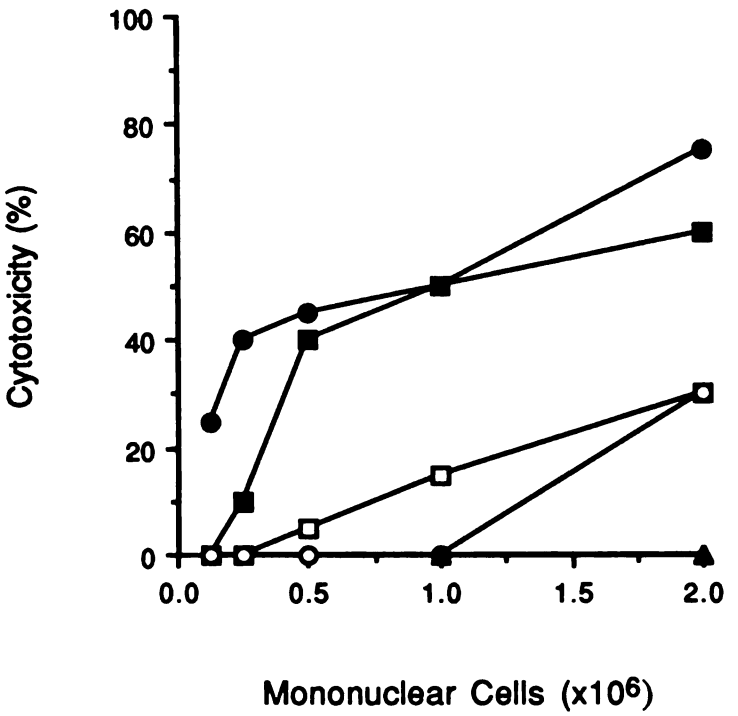


Figure 10. Antibody-dependent cell-mediated cytolysis (ADCC) of cell lines. (■) human NCI H69 in the presence of anti-Gal (20μg/ml); (□) NCI H69 in the absence of anti-Gal; (●) human MG 63 in the presence of anti-Gal; (○) human MG 63 in the absence of anti-Gal; (▲) human H498 or hamster CHO cells in the presence of anti-Gal.

The ability of transformed human cells to express α-Gal epitopes was assessed by RIA with anti-Gal and subsequently confirmed by rosetting analysis with BS-lectin and rabbit erythrocytes and by anti-Gal mediated ADCC. The results of these studies with a variety of human cell lines compared with other mammalian cell lines are presented in Table IV. Galili et al. (34) were not able to detect α-Gal epitopes on several human cell lines by BS-lectin binding. However, eight cell lines were found in the present study which could interact with the lectin at concentrations of less than 50μg/ml, as determined by a rosetting assay with rabbit erythrocytes. Cells of murine and New World monkey origin had the highest affinity for the lectin and formed rosettes at less than 1μg/ml. Once formed, rosettes could be disassociated by the addition of α-methyl galactoside. Since BS-lectin is known to interact specifically with α-Gal epitopes (83), this confirms that the rosetting was due to the presence of these epitopes on these cell lines. This lectin is a tetramer, allowing it to bridge between the α-Gal epitopes on the nucleated cells and the abundant epitopes on rabbit erythrocytes, resulting in rosette formation. The majority of human cell lines do not interact with the lectin and representative cell lines are shown in Table IV.

Some human cell lines bound BS-lectin quite strongly. In the case of SW1417, a cell line taken from a type B individual (79, 80), the rosetting endpoint was 1μg/ml. This is to be expected, since BS-lectin also interacts with the fucosylated, type B, terminal α-Gal epitope (83). Evidence that this is the type B blood group epitope is

provided by comparing the BS-lectin rosetting endpoint with the value for 50% inhibition of anti-Gal binding in the RIA. As seen in Table IV, anti-Gal did not interact with SW1417 cells. Since anti-Gal does not interact with the fucosylated α-Gal epitope, (27) it is likely that this epitope is the type B structure. Thus, cell lines which bound BS-lectin but not anti-Gal might represent cells expressing the blood group B structure and not the α-Gal epitope.

Several transformed human cell lines bound both BS-lectin and anti-Gal, confirming by two different means that these are α-Gal epitopes. To further ensure that the anti-Gal interaction was specific, RIA inhibition assays were performed with fixed cell lines, which were incubated with blood type AB plasma depleted of anti-Gal, to block potential F_c receptors. There was a 200-500 fold difference in the number of MG 63, ATB1 or NCI H69 cells required to inhibit 50% of anti-Gal binding in the RIA versus the number of murine NIH 3T3 or SP2/0 cells required for this inhibition. Based upon Scatchard analyses performed by Galili et al. (34) with murine SP/2 cells, these human cell lines may express a density of from 2000 to 10,000 α-Gal epitopes per cell.

The results of ADCC assays, as % killing in Table IV, showed that the ability of anti-Gal to interact with transformed human cells was correlated with the ability of the antibody to mediate cytotoxicity. There was one exception to this correlation. HT 144 cells bound neither the antibody or the lectin but the antibody could mediate ADCC. One possibility is that the ADCC assay is the more sensitive of the assays for detecting α-Gal epitope expression on cells. Thus, for very low numbers of α-Gal epitopes per cell (below 2000)

the RIA may not be sufficiently sensitive. Cells of type O origin, which included two colon carcinoma cell lines HM-7 and LS174T, were reported to have type B galactosyltransferase activity (79), and a breast carcinoma line, MCF-7 (81), interacted with BS-lectin but not with anti-Gal. The ADCC results suggest that anti-Gal can interact with these cells but that this may be below the level of detection of the RIA. Other cell lines, which could not interact with either the lectin or the antibody were not killed in the ADCC assay.

Table IV

Detection of α-Gal Epitopes on Human Cell Lines

Cell Line	Cell Type	BSB_4 Rosetting Endpoint[a]	50% Inhibition[b]	Killing[c] %
ATB1	EBV transformed B-cell	5.0	$4.0x10^6$	60
NCI H69	Small cell carcinoma, lung	2.5	$8.5x10^6$	70
MG 63	Osteosarcoma	>50	$3.0x10^6$	63
LS 174T	Colon carcinoma	6	$>10^7$	58
HM-7	Colon carcinoma	12	$>10^7$	40
BeWo(ATCC)	Choriocarcinoma	>50	$>10^7$	ND
SW1417	Colon carcinoma	1	$>10^7$	0
MCF7	Breast carcinoma	5	$>10^7$	33
MCF7 RAS	RAS + breast carcinoma	5	$>10^7$	47
COLO 205	Colon carcinoma	25	$>10^7$	0
KG1	Myelogenous leukemia	>50	$3.0x10^6$	70
HT144	Malignant melanoma	>50	$>10^7$	61
RAJI	Burkitt lymphoma	50	$>10^7$	ND
H4	Neuroglioma	>50	$>10^7$	0
HELA	Cervical carcinoma	>50	$>10^7$	5
CHO	Chinese hamster ovary	>50	$>10^7$	0
COS 1	SV40+ O. W. Monkey Kidney	50	$>10^7$	ND
SKO007J3	Human myeloma	>50	$>10^7$	ND
A498	Kidney carcinoma	>50	$>10^7$	0
HL60	Promyelocytic leukemia	>50	$>10^7$	0
SW 13	Adrenal adenocarcinoma	>50	$>10^7$	0
JAR	Choriocarcinoma	>50	$>10^7$	ND
HT1197	Bladder carcinoma	>50	$>10^7$	ND
Jurkat	T cell lymphoma	>50	$>10^7$	ND
Raji	Burkitt Lymphoma	>50	$>10^7$	ND
H498	Colon carcinoma	>50	$>10^7$	0
3T3	Mouse fibroblast	<1	$1.5x10^4$	ND
SP2/0	Mouse myeloma	<1	$1.5x10^4$	ND

[a] Rosetting analysis was performed incubating cell lines with BSB_4 lectin at various concentrations (in μg/ml), followed by pelleting cell lines with rabbit erythrocytes.

[b] RIA assays were performed with cell lines using anti-Gal. Data indicate the number of cells required to inhibit 50% of anti-Gal binding to antigen coated microtiter wells.

[c] Assays represent % cells killed by 10^6 lymphoctes plus anti-Gal vs controls with no anti-Gal. ND = not done.

α1,3GT Activity in Transformed Human Cell Lines. Since it was now apparent that some human cell lines could express low numbers of α-Gal epitopes, it was of interest to determine if the epitope expression could be correlated with α1,3GT activity in these cell lines. The activity of α1,3GT in solubilized microsomes of various human cell lines was assayed utilizing ^{3}H-UDP Gal as the substrate and LacNAc-Synsorb as the acceptor. The rate of incorporation of ^{3}H-α–Gal onto LacNAc-Synsorb is shown in Figure 11. Whereas the activity of the α1,3GT in murine L cell microsomes was high (approx. 670 pmol ^{3}H-α–Gal/g/hr), the activity seen with all human cell lines examined was marginal. The α1,3GT activity in the human cell line with the highest level, NCI H69, was at least 10-fold lower than the L cell activity. The levels of activity in microsomes of other human cell lines were within background errors for boiled, inactivated, microsomes and are below the level sensitivity of this assay.

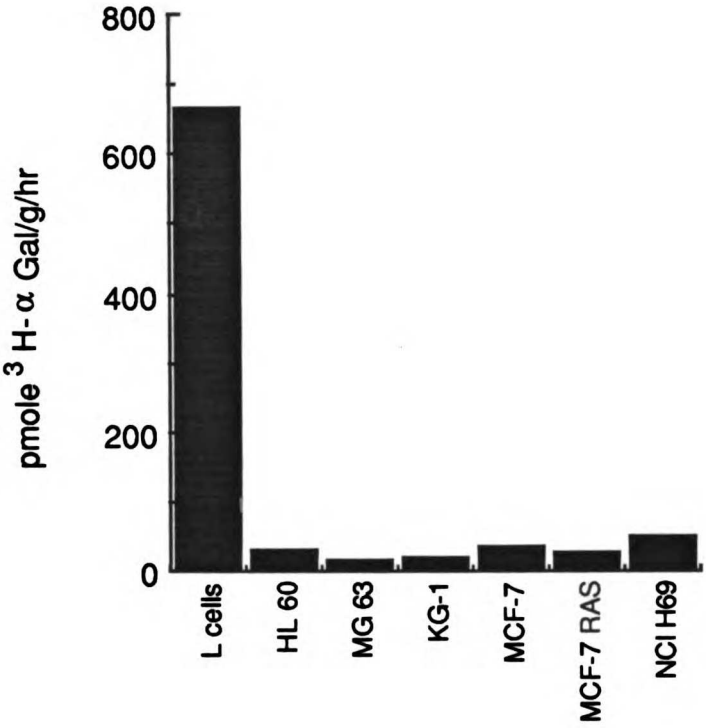


Figure 11. α1,3GT activity in human cell lines compared with the activity in mouse L cells. Solubilized cell microsomes were incubated with Sysorb-LacNAc beads in the presence of ^{3}H-UDP Gal, as described in Materials and Methods. Values represent specific release of ^{3}H Gal from Synsorb beads by α-galactosidase.

Transfection of HeLa Cells with a Murine α1,3GT cDNA. To determine whether human cells have a mechanism which inherently prevents high level α1,3GT expression, HeLa cells, which do not express α-Gal epitopes (see Table IV), were transfected with a a plasmid containing the murine α1,3GT cDNA (pCDM7-α1,3GT). This plasmid, which encodes a functional enzyme (59, 110), was used for a calcium phosphate co-transfection of HeLa cells, along with a plasmid containing the neomycin phosphotransferase gene (pCDM7-neo), as described in Materials and Methods. The murine α1,3GT cDNA contains 276 nucleotides upstream of the translational initiation site. Thus, this cDNA may also contain regulatory sequences for the expression of the α1,3GT gene.

HeLa cells co-transfected with the plasmid pCDM7-α1,3GT and with a pCDM7-neo were selected for resistance to G418 (a synthetic neomycin analogue). HeLa cells transfected with only pCDM7-neo were used as control cells. G418 resistant HeLa cells which were transfected with pCDM7-α1,3GT were subjected to two sequential rounds of selection by FACS, as described in Materials and Methods, utilizing biotinylated BS lectin as a means for the detection of α-Gal epitopes. Following the two sequential FACS selections, transfected HeLa cells were incubated with 25μg/ml BS lectin, followed by washing and incubation with rabbit erythrocytes. Approximately 55% of these FACS selected HeLa cells could form rosettes with the rabbit erythrocytes. In addition, the binding of anti-Gal to selected HeLa cells transfected with pCDM7-α1,3GT was compared with the binding to the pCDM7-neo transfected HeLa cell controls by FACS

analysis (Figure 12). The FACS analysis shows a ten-fold increase in the mean fluorescence intensity of the selected HeLa cells versus the control G418 resistant cells.

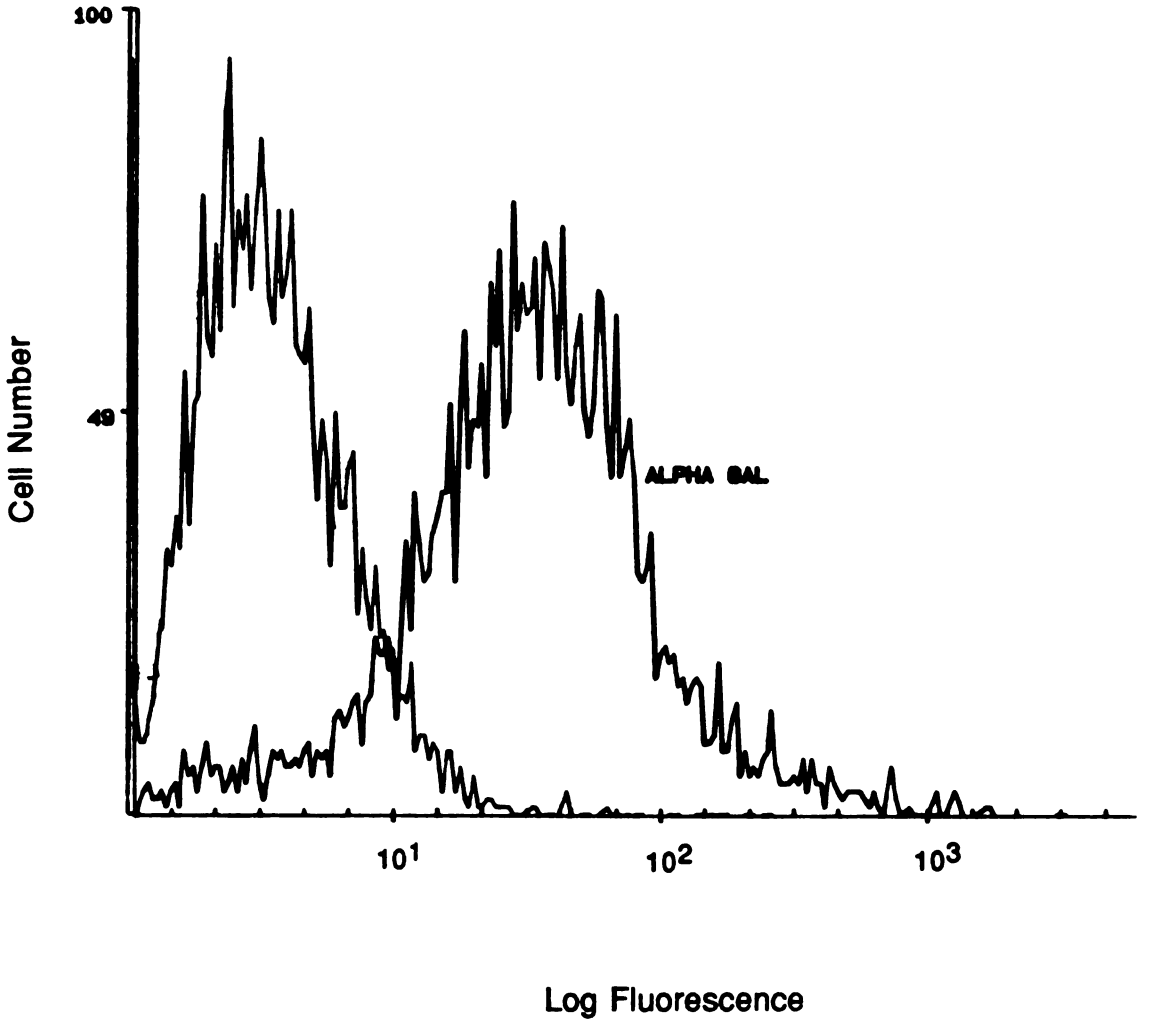


Figure 12. Flow cytometry analysis of α-Gal epitopes on FACS selected co-transfected HeLa cells versus control G418 resistant HeLa cells. Hela cells co-transfected with plasmids pCDM7-α1,3GT and pCDM7-neo are represented by the profile labelled "Alpha Gal". The unlabeled profile represents HeLa cells transfected with plasmid pCDM7-neo only. Hela cells were transfected and selected by FACS sorting as described in Materials and Methods. Cells were incubated with biotinylated anti-Gal for 1h, followed by washing and staining with FITC-avidin.

As was shown earlier in this chapter, transformed human cell lines expressing α-Gal epitopes can undergo anti-Gal mediated ADCC in the presence of normal human mononuclear cells. Therefore, the ability of the FACS selected HeLa transfectants expressing α-Gal epitopes to undergo ADCC compared with the G418 resistant controls transfected with pCDM7-neo only was studied. As shown in Figure 14, up to 74 % cell killing was observed in the HeLa transfectants expressing α-Gal epitopes but no significant killing was observed in the control, G418 resistant, HeLa cells.

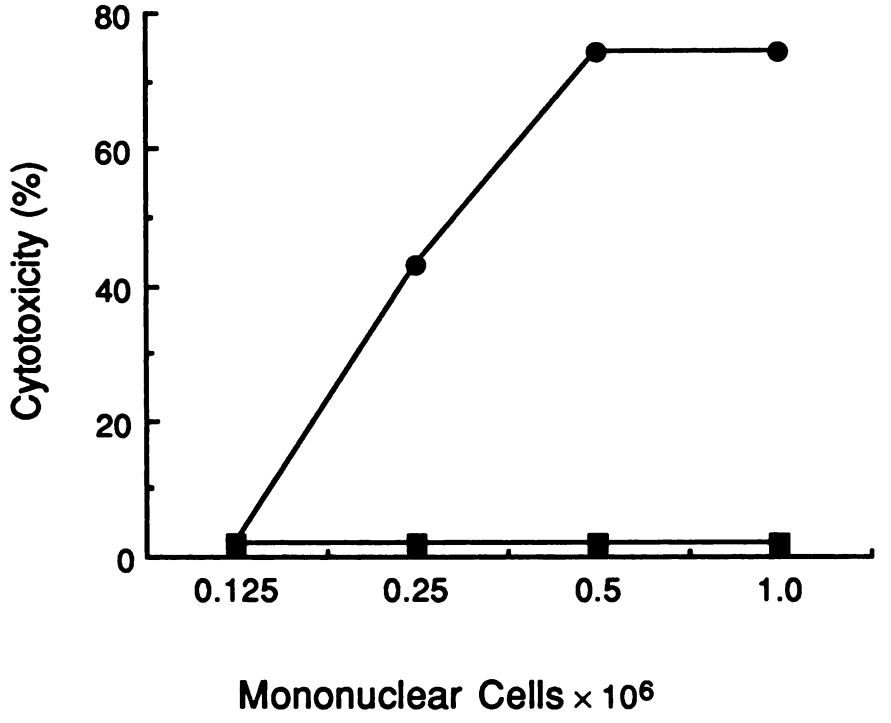


Figure 13. Antibody-dependent cell-mediated cytolysis (ADCC) of transfected HeLa cell lines. (●) HeLa cells co-transfected with a murine α1,3GT cDNA and a G418 resistance gene which were selected by FACS sorting for α-Gal epitope expression; (■) HeLa cells transfected with the G418 resistance gene only. HeLa cells were plated in 24 well plates and human mononuclear cells were added for 24 hours in the presence or absence of anti-Gal (1:8 rabbit rbc agglutination titer). Cytotoxicity represents the HeLa cell killing in the presence of anti-Gal minus HeLa cell killing in the absence of anti-Gal.

Discussion

The studies in this chapter suggest that transformed human cells have the capacity for the synthesis of α–Gal epitopes. Based upon RIA's in this study, and data on the number of these epitopes per cell on murine SP/2 cells provided by Galili et al. (34), the number of α–Gal epitopes on human cells expressing these epitopes is within a range of 2,500 to 10,000. The specific interaction of anti-Gal with these cells was demonstrated by pretreatment with α-galactosidase. This resulted in a complete elimination of anti-Gal binding, confirming that α-Gal epitopes are involved in this interaction. Since anti-Gal preparations from three different individuals and from pooled AB plasma gave the same results with human cell lines in the RIA, it is likely that anti-Gal antibodies in most, if not all individuals, are capable of interacting with terminal α–Gal epitopes on malignant human cells.

In addition to established transformed cell lines obtained from the ATCC (American Type Culture Collection), it was also of interest to explore the potential for the induction of synthesis of α–Gal epitopes on human cells not originally expressing these epitopes. Several different strategies were employed. One method involved mutation of HL 60 or HeLa cells with 300 RAD of gamma radiation. Cells were obtained by sequential (FACS), which could bind anti-Gal subsequent to blocking F_c receptors. However, these mutations were not stable and with time in culture these cells reverted to a negative α–Gal phenotype. Therefore, these cells could not be subjected to

further analysis. A stable α–Gal epitope expressing cell line was isolated by a completely different methodology. Normal, freshly isolated human B lymphocytes were transformed by Epstein-Barr Virus (EBV). They were then selected for anti-Gal binding ability by sequential FACS sorting. One cell line was obtained after three FACS sorts, which stably expressed α–Gal epitopes. This cell line was designated ATB1 and the analysis of this cell line appears in Table IV. It remains unclear whether the expression of α–Gal epitopes by this cell line is due to EBV induced mutations of normal B lymphocytes or the selection for rare B lymphocyte clones expressing α–Gal epitopes *in vivo*. Studies of this phenomenon await a characterization of the human α1,3GT genomic region.

Our studies have suggested that expression of α–Gal epitopes, though not usually expressed on human cells, can be aberrantly expressed under certain circumstances. It is not yet clear whether the synthesis of these epitopes is due to an aberrant expression of the mammalian α1,3GT or the activity of a "promiscuous" glycosyltransferase. The resolution of this issue required a molecular analysis of the activity responsible for synthesizing these α–Gal epitopes. In order to facilitate this molecular analysis, as described in Materials and Methods, an attempt was made to sequence the bovine α1,3GT and ultimately to clone the bovine α1,3GT gene. The α1,3GT enzyme was purified approximately 100,000 fold from bovine thymus tissue and an attempt at peptide sequencing was made. The preliminary attempt at sequencing failed, due to a blocked N-terminus. The strategy involved the utilization of the peptide sequence to synthesize degenerate DNA primers, which could

then be used to amplify a cDNA encoding the bovine α1,3GT gene. This bovine cDNA would then be used to study the expression of this gene in human cells aberrantly expressing α–Gal epitopes. However, before we could sequence and clone the bovine α1,3GT gene, it was cloned by Joziasse et al. (60). Simultaneously, the murine α1,3GT gene was cloned by Larsen et al. (59) and has been found to be homologous with a human genomic sequence (62). Larsen et al. found that the human homologue has two frameshift mutations within the coding sequence. Dr. Galili has since confirmed the presence of these two frameshift mutations in the MG 63 and ATB1 cell lines and could not detect the presence of α1,3GT mRNA by PCR analysis (personal communication). Joziasse et al. (60) found no expression of α1,3GT mRNA in transformed human cell lines, and thus, suggested that the suppression of the activity of this enzyme occurs at the transcriptional level. This raises the possibility that the α1,3GT activity seen in the MG63 and ATB1 cell lines is due to the activity of a "promiscuous" glycosyltransferase. An unpublished study by Galili of the fusion of murine SP2/0 cells with human peripheral blood lymphocytes, showed that these fused cells retain the ability to synthesize α-Gal epitopes. This suggested that human cells do not have a negative transcriptional regulatory factor, which could suppress the synthesis of α1,3GT mRNA. Therefore, the suppression of the synthesis of α1,3GT mRNA in human cell might result from a mutation in the transcriptional regulatory region of the α1,3GT gene. These studies prompted us to investigate the ability of human cells to translate the mammalian α1,3GT mRNA. To address this issue, HeLa cells, which do not normally express α-Gal epitopes,

were transfected with a plasmid containing a functional murine α1,3GT cDNA, containing a large upstream untranslated sequence. Indeed, as FACS analysis of anti-Gal staining and the interaction with BS-lectin suggested, transfected HeLa cells were selected which could express α-Gal epitopes. Therefore, it is unlikely that human cells possess a factor which can inhibit the translation of α1,3GT mRNA. In addition, a biological outcome of the expression of this gene in human cells was investigated by utilizing an ADCC assay. The majority of FACS selected HeLa cells transfected with the α1,3GT cDNA were killed in this assay when anti-Gal was added, while cells transfected with a control neomycin resistance plasmid were not killed in the presence of anti-Gal.

An attempt was made to quantify the level of α1,3GT activity in human cell lines and compare these cells with a well characterized cell line, murine L cells. L cells were found to have at least $6x10^6$ α-Gal epitopes per cell (34) and, as suggested in studies in this chapter, human cell lines expressing these epitopes have 2,500 to 10,000 per cell. If there is a direct correlation between the number of epitopes per cell and the level of α1,3GT activity, then there would be expected to be a difference of 10^3 to 10^4 in the level of enzyme activity in human versus murine cells. Thus, it is not surprising that measurable levels of α1,3GT activity could not be detected in microsomes of transformed human cell lines expressing low numbers of α-Gal epitopes.

The ability to synthesize small numbers of the α-Gal epitopes on human cells may have important consequences for the generation of an autoimmune response. ADCC results presented in this

dissertation suggest that the interaction of anti-Gal with these epitopes on human cell lines is sufficient for triggering a cytotoxic immune response against these cells. *In vivo*, such α-Gal epitope expression on cells might result in an anti-Gal mediate autoimmune response directed at these cells. The threshold level of α-Gal epitope expression required for this autoimmune response might be below 2,500 epitopes per cell, since ADCC was demonstrated at epitope levels too low to detect by the sensitive RIA.

The studies of α-Gal epitope expression on transformed human cell lines have provided support for the potential role of anti-Gal in autoimmune processes. It has also been suggested by Galili et al. (66), that exposure of cryptic α-Gal epitopes on pathological and aged red blood cells might contribute to their immune mediated destruction. Another physiological involvement of these epitopes on tumor cells in humans might be, as was suggested by Castronovo et al. (61), a part of a natural immune defense against cells with aberrant gene regulation. According to these investigators, the α-Gal epitope is expressed on malignant cells of half of patients with mammary carcinomas. The interaction of anti-Gal with such malignant cells was speculated to interfere with the metastatic potential of these cells.

Since these studies established that α-Gal epitopes can be aberrantly expressed on human cell lines, it was of interest to observe whether such a phenomenon could be observed *in vivo*. One organ, which in humans has been suggested to be involved in anti-Gal mediated autoimmune diseases, is the thyroid (21). As will be shown in Chapter V, experiments were designed to study the

synthesis of the α-Gal epitope in thyroid cells of mammalian and human origin, in a comparative manner.

CHAPTER V

The α–Galactosyl Epitope on Mammalian Thyroid Cells

Introduction

Studies in Chapter III suggested that there is an evolutionary suppression of α-Gal epitope synthesis on secreted glycoproteins in humans and Old World monkeys. When transformed human cell lines were examined for α-Gal epitope expression in Chapter IV, several cell lines were found which expressed these epitopes. Preliminary studies by Galili et al. (34) suggested that cell lines of human and Old World monkey origin might not normally express α-Gal epitopes. However, there has been no information on α-Gal epitope synthesis and expression *in vivo* in various species. Therefore, it was of interest to examine the evolutionary pattern of expression of these epitopes on cells and their biosynthesis by the α1-3galactosyltransferase (α1,3GT) in one tissue, obtained from various mammals. The α1,3GT catalyzes the following reaction in the Golgi apparatus:

Galβ1→4GlcNAc-R + UDP-Gal ————→ α-Galβ1→4GlcNAc-R + UDP.

Whereas α1,3GT activity has been demonstrated in murine Ehrlich ascetes tumor cells (53, 55), rabbit stomach mucosa (57), bone marrow cells (56), and cow thymus cells (58), it was not detected in human cell lines by Galili et al. (34). The α1,3GT activity of transformed cell lines was also below the level of detection of the

enzymatic assay utilized in Chapter IV. Since the expression of these epitopes has been characterized on thyroglobulin, the thyroid was chosen as a model tissue for these studies. In this chapter, we have also correlated the α1,3GT activity with the actual amount of α-Gal epitopes produced. The expression of α-Gal epitopes was carried out by using the sensitive RIA developed in Chapter III. The activity of the α1,3GT in these cells was measured by the two assays, as described in the Materials and Methods section (part L). As will be discussed, these studies suggest that the expression of α-Gal epitopes on mammalian thyroid cells does not correlate with α1,3GT activity in these cells. In addition, the studies in this chapter demonstrate for the first time the evolutionary pattern of α-Gal epitope expression on cells *in vivo*.

Results

α1,3GT activity in thyroid microsomal fractions. Thyroid microsomes obtained from various species were first assayed for α1,3GT activity by measuring specific transfer of [^{3}H] Gal to LacNAc-R Synsorb in an α-anomeric linkage. As seen in Figure 14, the α1,3GT activity in all species, except for rhesus monkey and human thyroid microsomes, was within the same order. Microsomes from cow thyroid displayed the highest activity among nonprimate mammals studied. The enzyme activity in cow thyroid was only nine-fold higher than that in guinea pig thyroid, which was the lowest among nonprimate mammals. Among primates, enzymatic activity linking α-[^{3}H] Gal to LacNAc-R acceptors was readily demonstrable in squirrel monkey (a

New World monkey) thyroid microsomes, but was completely negative in rhesus monkey (an Old World monkey) thyroid and in human thyroid. The contribution of β-galactosyltransferases to the linkage of radiolabeled galactose to the solid-phase acceptor was minimal in all species, as indicated by the marginal release of [^{3}H] Gal, mediated by β-galactosidase (Fig. 14).

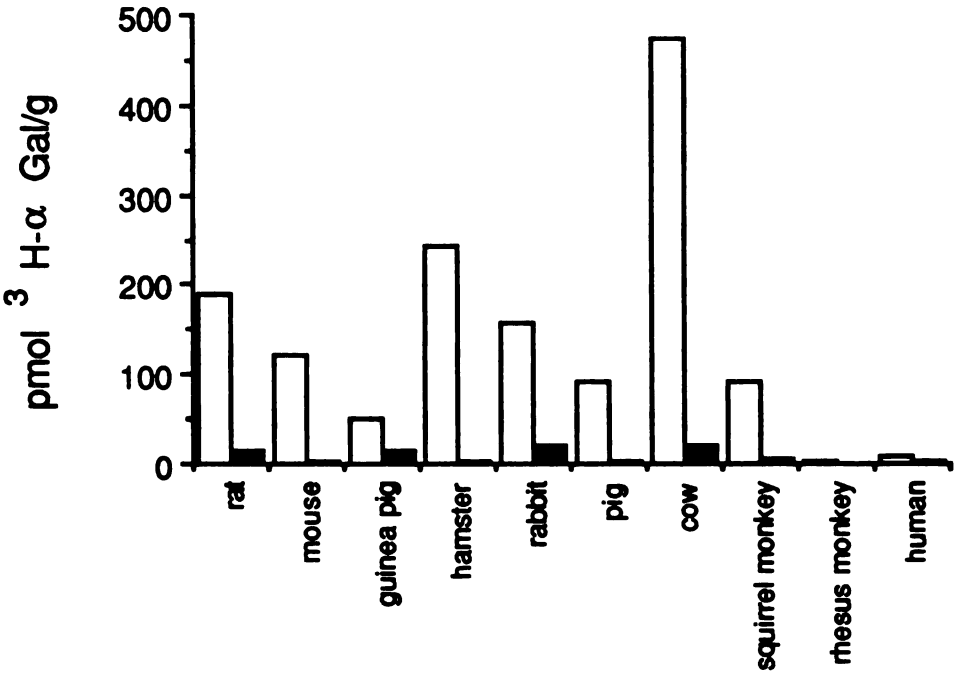


Figure 14. Assessment of microsomal α1,3GT activity linking α-galactosyl epitopes to LacNAc-R Synsorb beads. Open columns, [^{3}H]-Gal released by α-galactosidase; closed columns, [^{3}H]-Gal released by β-galactosidase.

The distinct activity of α1,3GT in microsomes of nonprimate mammals and New World monkey thyroids was further confirmed in an assay where the *de novo* synthesized α-Gal epitopes could be detected by the monoclonal antibody, Gal-13, which is highly specific for this structure (48) (Fig. 15). The solid-phase acceptor in this assay was Galβ1→4GlcNAc-R on fixed rabbit red blood cells (rrbc) attached to microtiter wells, which were pretreated with α-galactosidase (see Materials and Methods). Thyroid microsomes from all the nonprimate mammals and the New World monkey synthesized α-Gal epitopes on LacNAc-R acceptors of the rrbc. These epitopes subsequently bound the Gal-13 monoclonal antibody. Gal-13 bound to microtiter wells was detected by incubation with a biotinylated anti-mouse IgG followed by incubation with ^{125}I-streptavidin. When this reaction was performed with Old World monkey and human thyroid microsomes, only minimal binding of Gal-13 was observed, indicating a diminished α1,3GT activity in these species. It should be stressed that identification of α-Gal epitopes with Gal-13 could not be performed utilizing LacNAc-R Synsorb beads as α1,3GT acceptors, since ^{125}I-streptavidin used in the last step of this assay displays a high, nonspecific binding to Synsorb beads. In contrast, we observed only minimal binding of ^{125}I-streptavidin to the rrbc. In addition, the α1,3GT activity in mouse microsomal preparations could not be assessed in the rrbc assay, since the contaminating mouse IgG in the these preparations resulted in a high background of biotinylated anti-mouse IgG antibody binding.

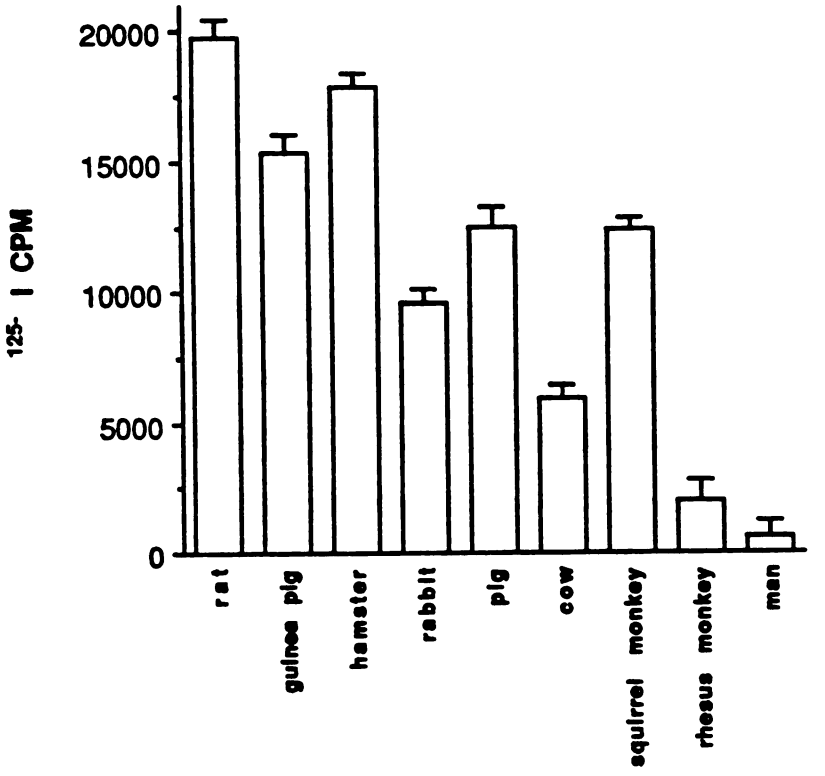


Figure 15. α1,3GT assay with microsomal fractions obtained from thyroid glands of various species. Fixed rabbit red cells pretreated with α-galactosidase served as acceptors. Synthesis of α-Gal epitopes was identified by binding of the monoclonal antibody Gal-13 and subsequent binding of biotinylated anti-mouse IgG and ^{125}I-streptavidin. Mean ± SE of data obtained from triplicate assays.

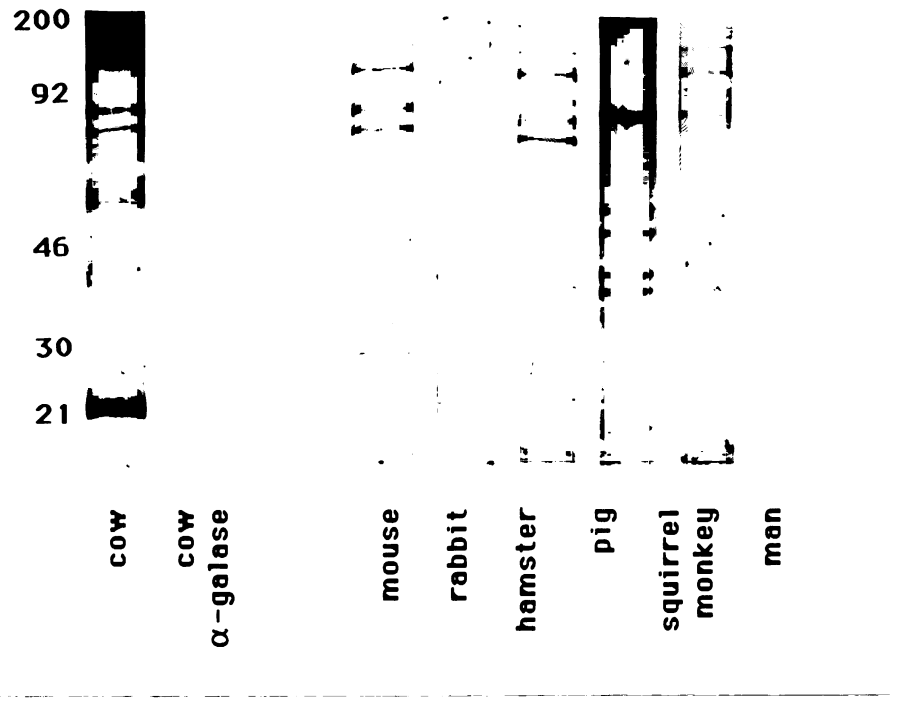
α-Gal epitopes on thyroid cell membrane glycoproteins. The demonstration of α1,3GT activity in thyroids of nonprimate mammals and New World monkey prompted us to identify the biosynthetic product of this enzyme (i.e., the α-Gal epitope) in thyroid cell membrane glycoproteins of these species.

Membrane fractions of thyroid cells from various species were solubilized with Triton X-100, adjusted to equal protein concentrations, processed for Western blot analysis, and immunostained with biotinylated anti-Gal. Many membrane glycoproteins in cow thyroid expressed this epitope (Fig. 16). A distinct glycoprotein with a molecular weight of approximately 20,000 had a large number of these epitopes, and was thus heavily stained by anti-Gal. This glycoprotein was previously isolated by Okada and Spiro (91) and designated GP-3. Carbohydrate analysis of this molecule revealed an abundance of the α-Gal epitope (45). Pretreatment of the cow thyroid membrane glycoprotein preparation with α-galactosidase prior to gel electrophoresis resulted in the elimination of anti-Gal binding to the blotted glycoproteins. Pretreatment with β-galactosidase had no effect upon the immunostaining pattern compared with the original preparation (not shown).

Anti-Gal staining of glycoproteins from thyroid cell membranes of other mammalian species suggested that the concentration of the α-Gal epitopes on membrane glycoproteins is highly variable. These epitopes were abundant in pig cell membrane glycoproteins, and in much lesser amounts in rabbit, rat, hamster, guinea pig, and squirrel

monkey. Pretreatment of these preparations with α-galactosidase resulted in the elimination of anti-Gal binding (not shown), as exemplified with cow thyroid glycoproteins. Glycoproteins from human thyroid also lacked the α-Gal epitopes, as indicated by the negative immunostaining with anti-Gal (Fig. 16).

Figure 16. Western blot and anti-Gal immunostaining of membrane glycoproteins from thyroid glands of various mammalian species. In the second lane, cow glycoproteins were pretreated with α-galactosidase (α-galase).


200
92
46
30
21
cow
cow
α-galase
mouse
rabbit
hamster
pig
squirrel
monkey
man

To quantify α-Gal epitope expression on thyroid cell membrane glycoproteins which displayed weak anti-Gal staining, we used an RIA we developed for this purpose (95). It is interesting that cow thyroid glycoproteins were reactive in the RIA to the same extent as cow thyroglobulin (Fig. 17). This implied that the concentration of α-Gal epitopes per mg of membrane glycoproteins in the cow thyroid is similar to that in the secretory product of the cells, i.e., thyroglobulin. Based on the measurements of Spiro and Bhoyroo (6), the concentration of these epitopes on cow thyroid membrane glycoproteins is 16 nmole/mg. Other nonprimate species had lower concentrations of these epitopes, ranging between 6 nmole/mg in pig thyroid and 0.2 nmole/mg in hamster (Fig. 17, Table V). Analysis of primate thyroid glycoproteins demonstrated 2 nmole/mg of α-Gal epitopes in squirrel monkey thyroid. In accord with the diminished α1,3GT activity in rhesus monkey and human thyroids, no expression of the α-Gal epitope was measured in solubilized thyroid membranes obtained from these species.

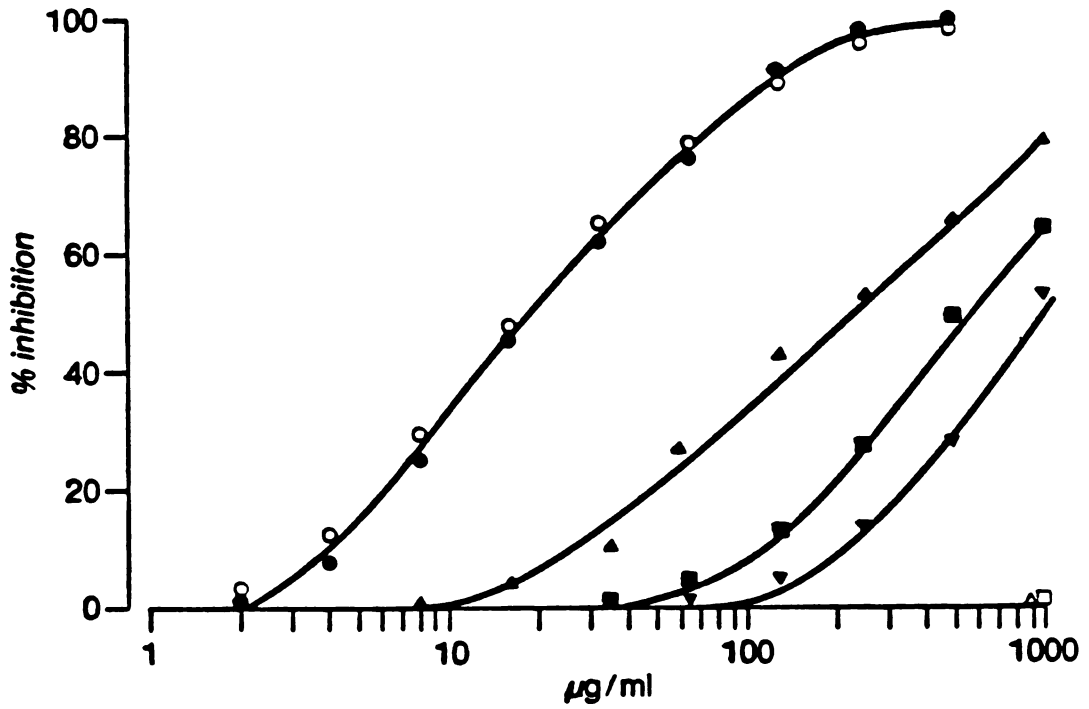


Figure 17. Solid-phase RIA based on inhibition of anti-Gal (1 μg/ml) binding to cow thyroglobulin (enriched on BS–Sepharose) subsequent to preincubation of the antibody with glycoprotein preparations obtained from thyroid glands of: ● cow; ■ rat; ▼ rabbit; ▲ squirrel monkey; Δ rhesus monkey, and □ human origin. Cow thyroglobulin, O, was used as standard glycoprotein with known amount of α-Gal epitopes (16 nmole/mg).

Table V

Quantification of α-Gal epitopes on thyroid cell membrane glycoproteins

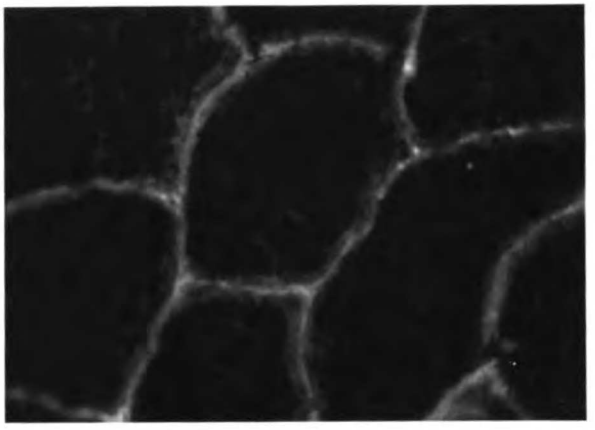
Species	Conc. of 50% Inhibition (μg/ml)	*nmole epitopes per mg protein
Mouse	1000	0.3
Rat	500	0.6
Guinea pig	1500	0.2
Hamster	1500	0.2
Rabbit	1000	0.3
Pig	50	6.0
Cow	20	16.0
Squirrel monkey	125	2.3
Rhesus monkey ◊	No inhibition	—
Man ◊	No inhibition	—

* Calculations are based on the study of Spiro and Bhoyroo (1984), who reported that bovine thyroglobulin contains 16 nmole/mg of α-Gal epitopes.

◊ No inhibition of anti-Gal binding at protein concentration of 1000 μg/ml.

In order to obtain information regarding the histology of α-Gal epitope expression in the mammalian thyroid, a frozen section from bovine thyroid was stained by incubation with biotinylated anti-Gal, followed by FITC-avidin. The fluorescence micrograph shown in Figure 18 shows a distinct staining of the thyroid follicular cells, surrounding the thyroglobulin containing colloid. Some staining of the thyroglobulin can also be seen. However, since thyrogloblin is water soluble, much of this glycoprotein was removed during the washing steps of anti-Gal binding assay. These results correlate with the studies of the α-Gal epitope and α1,3GT enzyme in this chapter, and also with the study of Spiro and Bhoyroo (6) on the presence of these epitopes on bovine thyroid cells.

Figure 18. Anti-Gal binding to a bovine thyroid frozen section. Biotinylated anti-Gal was incubated with a bovine thyroid 10μm frozen section, followed by incubation with avidin-fluorescein, as described in Materials and Methods.



Discussion

The data in the present study indicate that the evolutionary distribution of α1,3GT activity and its biosynthetic product in mammals, previously determined with various cell lines (34), is also applicable to tissues *in vivo*. α1,3GT activity was demonstrated by two independent assays in microsomal fractions from thyroid cells of all nonprimate mammals studied and from the New World monkey, squirrel monkey. However, this activity was not detected in thyroid cell microsomes of the Old World monkey, rhesus monkey, and of man. Whereas the assay measuring Gal-13 binding to α-galactosidase-treated rabbit red cells provided a qualitative measure for the *de novo* synthesis of α-Gal epitopes, measuring the α-linkage of [^{3}H] Gal to LacNac-R Synsorb provided the quantitative assessment of α1,3GT activity. Measurement of this enzyme activity in all species which synthesize the α-Gal epitope demonstrated at most a 9-fold difference in activity. In contrast, large variations in the expression of the α-Gal epitope were observed among various mammals using RIA. The concentration of this epitope in cow thyroid membranes was found to be 80-fold higher than in hamster or guinea pig thyroid membranes. These findings are further supported by the observation of large variations in the intensity of anti-Gal staining of thyroid cell membrane preparations. These findings suggest that in species producing α1,3GT *in vivo*, there is no correlation between α1,3GT activity in the Golgi apparatus and the

expression of the biosynthetic product of this enzyme on the cell membrane.

The *in vivo* variations in the α-Gal epitope expression differ from the pattern observed in cell lines. Quantification of the same epitope on cell lines demonstrated no significant difference in the number of epitopes per cell when the cell lines were derived from mouse, rabbit, pig, or cow (70). In addition, studies in a mouse teratocarcinoma cell line showed that the retinoic acid induced alteration in α-Gal epitope expression on cell membranes correlated with changes in α1,3GT activity within the Golgi apparatus (44), and with the rate of expression of the α1,3GT gene (59). The present study suggests that, *in vivo*, additional factors may participate in controlling the extent of α-Gal epitope synthesis on membrane glycoproteins. It is possible that variable competition between α1,3GT activity and sialyltransferases for the LacNAc acceptor is one of these factors (92). Whereas α1,3GT activities in pig and hamster thyroid microsomes do not differ significantly form each other, effective competition by sialyltransferases in the latter species would result in linkage of a terminal sialic acid to a large proportion of LacNAc acceptors rather than the α-Gal linkage.

Another factor which may affect the biosynthetic expression of α1,3GT is the branching of the carbohydrate chain on the glycoprotein molecule (i.e., the number of LacNAc epitopes branching from mannosyl core epitopes of N-glycosylated glycoproteins). The biosynthesis of di-, tri- and tetra-antennary glycoconjugates is dependent on the activity of a variety of GlcNAc transferases synthesizing core epitopes on these mannosyl epitopes

(See review of Schachter and Roseman, 94; Kornfeld and Kornfeld, 1). An example for such a multi-antennary glycoprotein is GP3 from cow thyroid cell membrane (91, 45) with a molecular weight of approximately 20,000. This glycoprotein has only two N-glycosylation sites, however there seem to be multi-antennary branches of each of the carbohydrate chains which result in a cluster of α-Gal epitopes previously demonstrated biochemically (45). Therefore, the intense immunostaining of a band of approximately this molecular weight, seen on the Western blot of cow thyroid glycoproteins, might be due to such a clustering of α-Gal epitopes.

Studies in this chapter indicate that α-Gal epitopes are synthesized on mammalian thyroid cells. However, the synthesis of these epitopes on human thyroid cells seems to be suppressed. Preliminary studies by Etienne-Decerf et al. (21, 69) suggested that α-Gal epitopes might be expressed on human pathologic thyroid cells. Therefore, we increased the sensitivity of the RIA for the detection of these epitopes by using fixed thyroid membrane fragments. In the next chapter, such assays of α-Gal epitope expression on normal and autoimmune human thyroid will be described.

CHAPTER VI

The α-Gal Epitope on Human Normal and Autoimmune Thyroid Cells: Possible relationship to autoimmunity

Introduction

Evidence has been accumulating which suggests that human cells have the potential for synthesizing α-Gal epitopes. This hypothesis was given support by studies in Chapter IV, which showed that some transformed human cells can aberrantly express α-Gal epitopes. Support for the potential of human cells to synthesize these epitopes also comes from observations that malignant cells from a substantial proportion of patients with mammary carcinomas and from human malignant cell lines seem to synthesize this epitope (15, 61). The α-Gal epitope was also found on human placental and tissue plasminogen activator (28). In addition, human red cells were found to have 1,000 to 2,000 cryptic α-Gal epitopes, which may be exposed upon red cell aging. These observations, along with the data on the presence of an homologue of the mouse α1,3GT gene in humans, led Galili to hypothesize that the interaction of the anti-Gal antibodies with aberrantly expressed α-Gal epitopes in human tissues might lead to autoimmune processes (70). One autoimmune disorder in which possible involvement of the anti-Gal and α-Gal epitopes was suggested is Graves' disease, an autoimmune disorder of the thyroid. Etienne-Decerf et al. (21, 69)

observed a marked increase in anti-Gal titers in patients with Graves' disease. These investigators also found low amounts of α-Gal epitopes on cell membrane glycoproteins obtained from the thyroid of a patient with Graves' disease (69), and showed anti-Gal binding to trypsin-treated normal human thyroid cells by immunofluorescence (21).

The objectives addressed in this Chapter were: 1.) The determination of the potential for the expression of α-Gal epitopes on human thyroid cells; 2.) To determine whether the expression of this epitope on thyroid cells of patients with Graves' disease differs from that of normal thyroids; 3.) The measurement of α1,3GT activity in microsomal fractions to determine the biosynthetic potential for α-Gal epitope synthesis in diseased and normal thyroid tissues; and 4.) To determine whether α–Gal epitope expression can occur in man in tissues other than the thyroid.

Results

Detection of α-galactosyl epitopes by RIA. The principles of RIA for α-Gal epitopes were described in the Materials and Methods section. Preliminary studies in Chapter V suggested that if α-Gal epitopes are present in the human thyroid, then they are present in low numbers. In order to detect low numbers of α-Gal epitopes on thyroid cell membranes, the sensitivity of the RIA was increased by quantification of this epitope on fixed membranes rather than on solubilized membrane proteins. This would result in a high concentration of the α-Gal epitopes, if present, in the assay system.

In accordance with previous observations that α-Gal epitopes are abundant on bovine thyroid cells (45), as few as 0.1 μl of packed bovine thyroid fixed membrane fragments (corresponding to 60μg of protein) were sufficient to yield a 50% inhibition of anti-Gal binding to solid-phase α-Gal epitopes (Fig. 19). Approximately the same level of interaction with anti-Gal was observed with fixed porcine thyroid membranes. The specificity of the assay could be further demonstrated by the decrease in inhibitory capacity of the membranes following pretreatment of fixed membranes with α-galactosidase. Membranes pretreated with β-galactosidase were as potent at binding anti-Gal as in the RIA of untreated membranes (not shown).

However, a different inhibition pattern was observed with human thyroid specimens. Thyroid membranes from one normal donor inhibited no more than 20% of anti-Gal binding to the solid-phase antigen in the RIA at a membrane volume of 100 μl , representing around 60mg protein (Fig. 19). The cell membranes obtained from one Graves' disease patient were more potent in the RIA, where 25 μl of thyroid membranes displayed 50% inhibition.

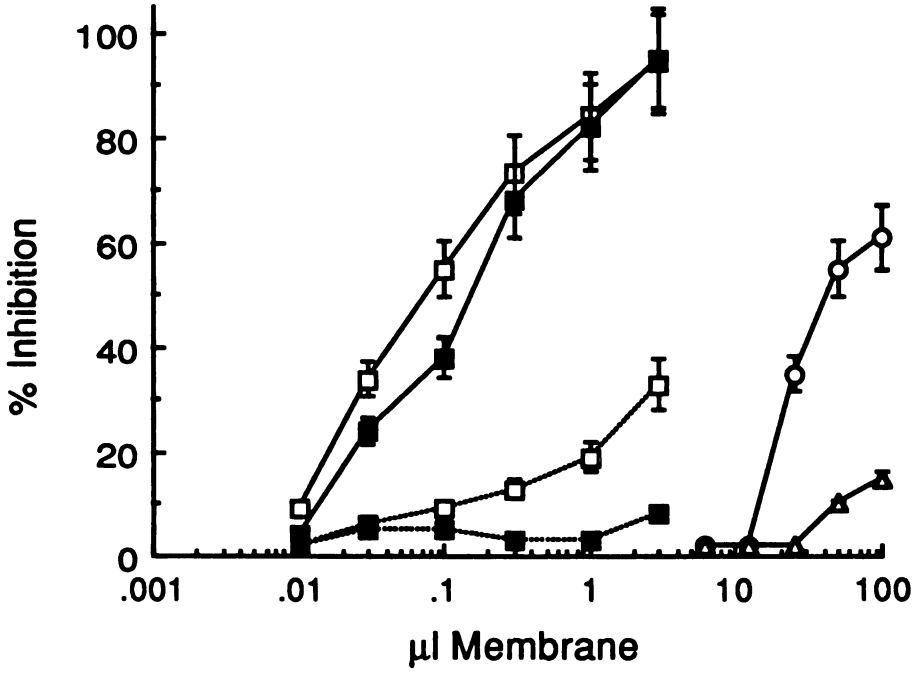


Figure 19. RIA with fixed membranes from: (■) bovine thyroid cells, dotted lines indicate membranes pretreated with α-galactosidase; (□) porcine thyroid cells, dotted lines indicate α-galactosidase pretreatment; (○) cells from a patient with Graves' disease; (Δ) normal thyroid cells. Values represent the mean and corresponding standard deviations from triplicate measurements.

α-galactosyl epitopes on human normal and diseased thyroid membranes. Since the preliminary data indicated an inhibitory effect of Graves' thyroid membranes in the RIA to be substantially greater than normal thyroid membranes, it raised the question of whether the aberrant expression of the α-Gal epitope is a characteristic of thyroid cell membranes of patients with Graves' disease. To address this question, RIA studies were performed with specimens obtained from patients with Graves' disease, nontoxic goiter, and individuals who died from causes other than thyroid disorders. The α-Gal epitope was found to be expressed at variable amounts in Graves' patients. Three out of six Graves' patients studied expressed this epitope at a level in which 100 μl of fixed membranes inhibited more than 50% of anti-Gal binding to the solid-phase antigen (Fig. 20). However, no patients with nontoxic goiter out of the four examined had thyroid cell membranes which inhibited anti-Gal at a level higher than 40%. Expression of α-Gal epitopes also was observed in individuals with no thyroid disorders. Five of the eleven specimens displayed inhibiting values higher than 50%. Specimens pretreated with α-galactosidase lost a large proportion of the inhibitory capacity in the RIA, whereas β-galactosidase did not alter the capacity of the membranes to inhibit RIA (not shown). Thus expression of the α-Gal epitope could be demonstrated both in thyroids of Graves' disease patients and in normal thyroids. These data imply that α-Gal epitope expression on thyroid cells is not a unique characteristic of the pathologic autoimmune tissue, but can be demonstrated in some normal tissues

as well. When expressed, however, the α-Gal epitope concentration on human thyroid cells is approximately 200- to 500-fold less than its concentration on bovine or porcine thyroid cells (Fig. 19).

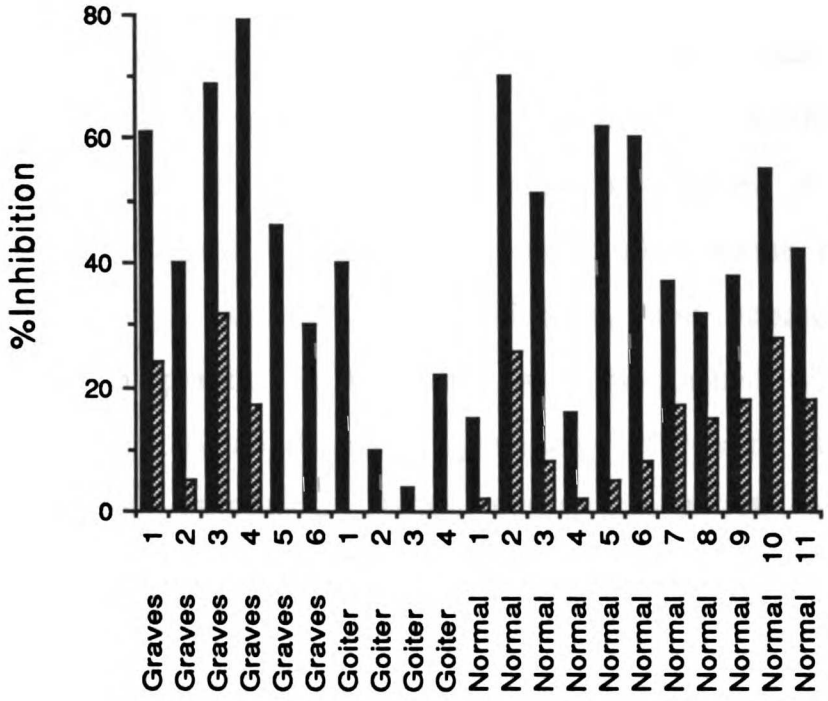


Figure 20. RIA with fixed thyroid cell membranes from normal individuals, patients with nontoxic goiter, and patients with Graves' disease. Filled bars: percent inhibition in the RIA with 100µl fixed membranes; hatched bars: percent inhibition in the RIA with 100µl fixed membranes pretreated with α-galactosidase. Specimens with no hatched bars were not assayed for the effect of α-galactosidase.

α-galactosyl epitopes on normal human kidney and liver. Since α-Gal epitopes were found to be expressed in normal as well as pathological human thyroid tissues, it was of interest to determine whether these epitopes are also expressed in other human tissues. Two tissues were chosen for this study, the kidney and liver. The kidney was chosen because it contains thick basement membranes containing laminin. Laminin has been shown in other mammals to have a high density of α-Gal epitope containing glycoconjugates (9, 25). Thus, if low numbers α-Gal epitopes are present in other human tissues, it would be of interest to begin to explore this by examining the kidney. The liver was chosen as another tissue to study because relatively large samples can be obtained and hepatocyte membranes are easily prepared. RIA studies were performed with fixed membranes from nondiseased specimens of human kidney and liver, recieved from autopsies. 100μl membrane pellets were examined from tissues taken from two normal individuals before and after pretreatment with α-galactosidase (Fig. 21). Whereas membrane preparations from both the kidney and liver of these two individuals could inhibit up to 50% of anti-Gal binding to the solid phase antigen, α-galactosidase pretreatment completely abolished this effect.

Since the anti-Gal prepared from pooled AB plasma could interact with the normal human kidney and liver membrane preparations, we were interested to confirm these results with three anti-Gal preparations from different blood group AB individuals. Similar results would suggest that this binding was not due to a contaminating antibody activity in the pooled anti-Gal preparation.

RIA assays with these human tissues were also run in parallel with assays of bovine tissues to compare the relative numbers of α-Gal epitopes. The same inhibition results were obtained from all three anti-Gal preparations. Representative inhibition curves are shown in Figure 20. The volume of fixed liver or kidney membrane required to inhibit 50% of anti-Gal binding in the RIA was approximately 100μl. In contrast, bovine kidney and thyroid showed 50% inhibitions at between 0.01 and 0.07μl of packed membrane, respectively. Bovine kidney membranes were especially potent at binding anti-Gal. This might be expected since bovine kidney contains laminin and other extracellular matrix molecules with large numbers of α-Gal epitopes (8). Furthermore, when human kidney or liver membranes were pretreated with α-galactosidase, the inhibition of anti-Gal binding to the solid phase antigen, BS^+ thyroglobulin, was completely abolished (Fig. 22). A substantial decrease in inhibition was also observed when bovine kidney membranes were pretreated with α-galactosidase. Similar results on the inhibition of anti-Gal binding in the RIA were observed with kidney and liver membrane preparations from another normal individual (not shown). Thus, anti-Gal which is capable of binding to α-Gal epitopes on human cells, is present in most, if not all, humans.

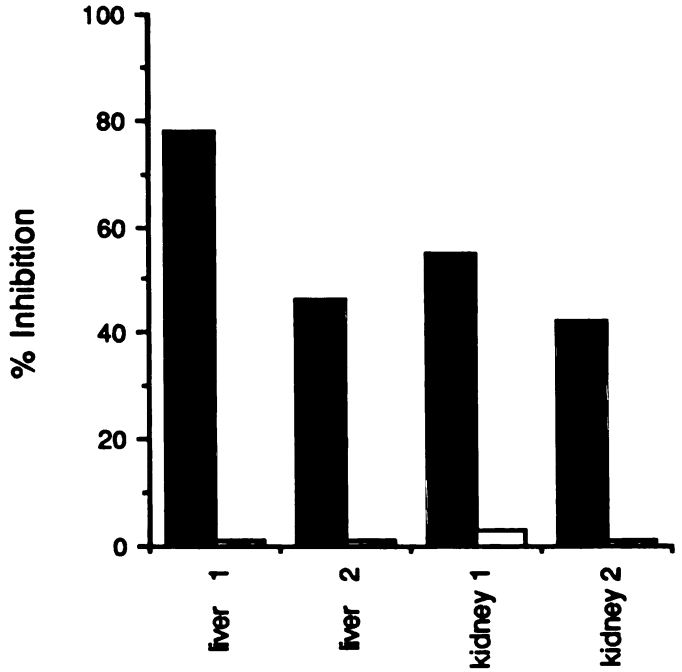


Figure 21. RIA with fixed membranes from normal human livers or kidneys. Filled bars: Percent inhibition in the RIA with 100µl fixed membranes; open bars indicate membranes pretreated with α-galactosidase. Bars with the same number represent membranes from the same individual.

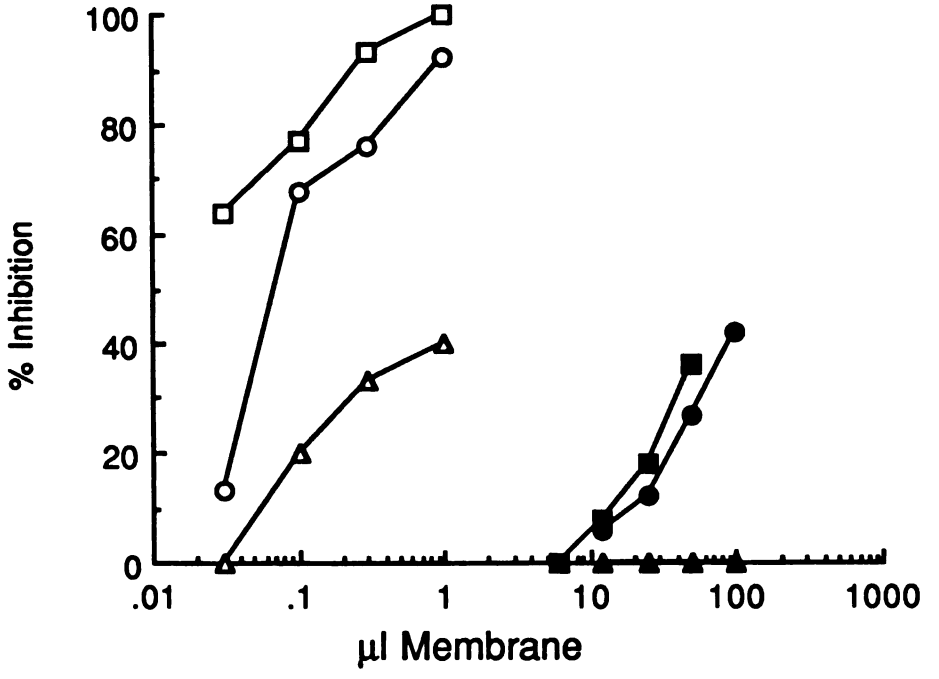


Figure 22. RIA assays with fixed membranes from: (□) bovine kidney cells; (○) bovine thyroid cells; (Δ) bovine kidney cell membranes pretreated with α-galactosidase; (■) human kidney; (●) human liver; (▲) human liver membranes pretreated with α-galactosidase. Curves represent identical RIA assay results obtained with anti-Gal, purified from AB plasma of three different individuals. Fixed membrane pellets were resuspended and mixed end-over-end during the incubation with anti-Gal.

α1,3GT activity in thyroid microsomes from normal donors and patients with Graves' disease. The finding of α-galactosyl epitopes on cell membranes of healthy individuals and of patients with Graves' disease raised the question of whether the enzymatic activity capable of synthesizing this epitope can be demonstrated in microsomal fractions from such tissue specimens. The assay for α1,3GT was based on measuring the α-linkage of [^{3}H] Gal to N-acetyllactosamine epitopes. The specificity of the assay in assessing the α-galactosyls linked to N-acetyllactosamine was established by measuring the release of the newly linked [^{3}H] Gal epitope with α-galactosidase. When microsomes from human normal thyroid specimens were assayed for α1,3GT activity, only four out of nine normal specimens displayed activity of more than 10 pmol/g/hr (Fig. 23a). Among Graves' disease patients, such activity was found in six of nine patients. Three Graves' microsome preparations had activities significantly greater than microsomes from normal thyroids. The mean α1,3GT activity in thyroids of Graves' patients (17 pmol/g/hr) seemed to be higher than that of controls (10 pmol/g/hr). However, this difference does not seem to be significant when one considers the range of the activities between the two groups. It should be stressed that due to limited amounts of pathologic thyroid tissues in some of the specimens, we could perform the enzyme assay but not the RIA for quantification of α-Gal epitopes.

Since α-Gal epitopes were observed in fixed normal human kidney and liver preparations it was of interest to determine

whether α1,3GT activity could also be detected in these tissues. As Figure 23b demonstrates, the level of α1,3GT activity in the normal thyroid and kidney microsomes examined were similar. However, the activity in normal liver was much higher than the normal thyroid or kidney. The activity observed in the liver microsomes was within the range of the Graves' disease microsomes with the highest α1,3GT activity. The α1,3GT activity in microsomes obtained from bovine thyroid tissue was assayed and directly compared with the activities in normal human thyroid, kidney or liver (Fig. 23c). The mean bovine α1,3GT activity was found to be 920 pmol/g/hr (i.e., more than 10-fold higher than that observed in human liver microsomes).

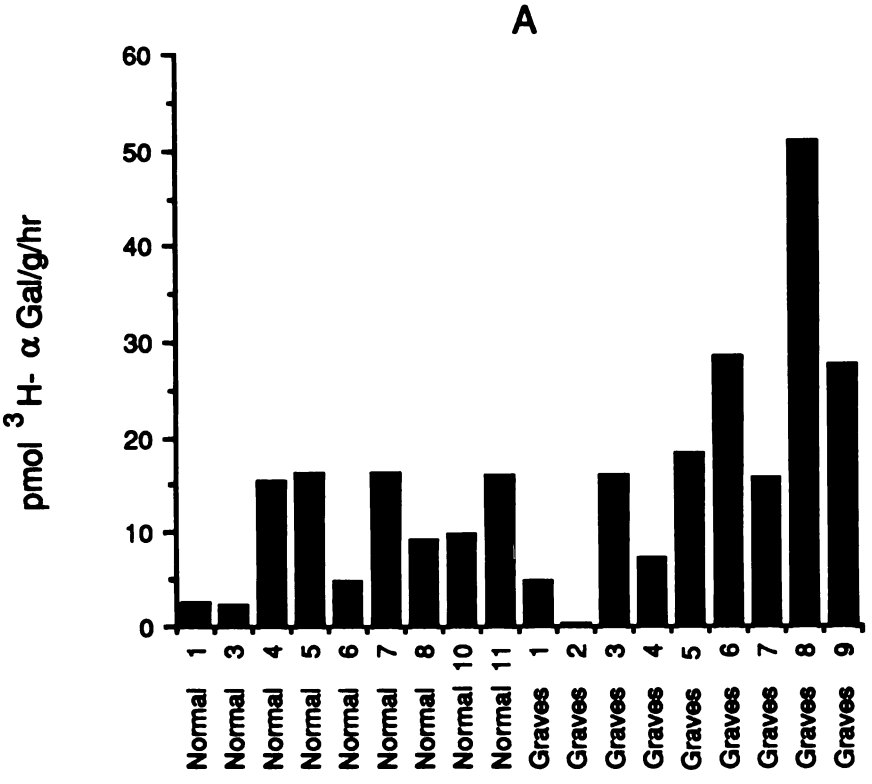
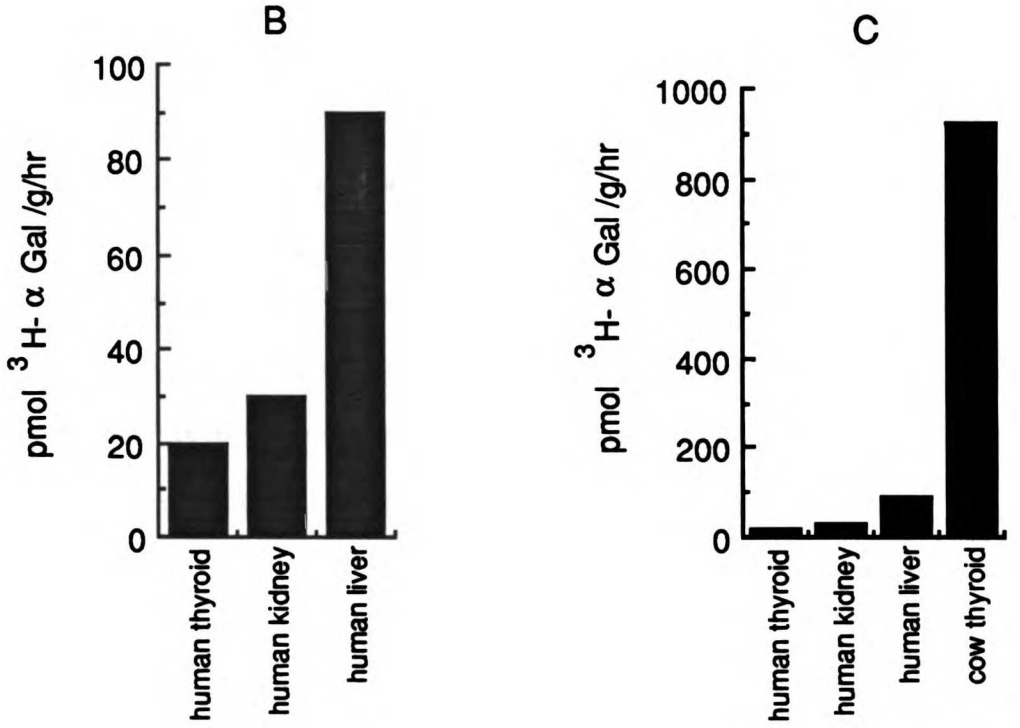


Figure 23. Activity of α1,3GT in microsomal fractions from cells of human tissues: (A) thyroid cells of normal humans and from patients with Graves' disease ; (B) a comparison of α1,3GT activity in normal human thyroid, liver and kidney microsomes ; and (C) a comparison of α1,3GT activities between microsomes from normal human tissues bovine thyroid microsomes.

Figure 23 (cont.)



Discussion

The most striking feature of the α-Gal epitope/anti-Gal system, as demonstrated in previous studies, is the diminished expression of this epitope in Old World monkeys, apes and humans, in comparison to its abundance in nonprimate mammals, prosimians and New World monkeys, and the reciprocal distribution of the natural anti-Gal antibody (33, 34, 99). The present study indicates, however, that when a highly sensitive method is used for detection of the α-Gal epitope, this epitope can be detected on thyroid tissues both from patients with Graves' disease and from individuals without apparent thyroid disorders. Furthermore, this expression is not limited to the thyroid, since normal kidney and liver tissues examined for α-Gal epitope expression, were also found to express these epitopes. While kidney and liver tissues from only two normal individuals could be obtained, this observation is nonetheless, interesting.

The occurrence of small amounts of α-Gal epitopes on human cells *in vivo* is further supported by observations of a low level of α1,3GT activity in microsomal fractions obtained from these tissues. These findings are unexpected in view of the large amounts of circulating anti-Gal antibodies which can interact with the α-Gal epitope.

The findings in this chapter suggest that, in spite of the evolutionary suppression, resulting in the loss of immune tolerance toward the α-Gal epitope, these epitopes are synthesized in human cells in low amounts. Since homogenized tissue preparations were

used for these studies, it is possible that these epitopes are normally sequestered and thus do not interact with the natural anti-Gal antibody *in vivo*, or that the low level of antigen–antibody interaction is of no biologic significance. Synthesis of very low amounts of α-Gal epitopes in man has been previously observed in red cells, where there are 1,000 to 2,000 such cryptic epitopes (65). These epitopes may be exposed upon aging of normal rbc, or may be exposed on pathologic rbc. The binding of anti-Gal to such epitopes may contribute to the opsonization of the senescent red cells for subsequent phagocytosis by reticuloendothelial macrophages (65, 66, 102). Low amounts of α-Gal epitopes in normal human tissues also have been suggested to occur by other investigators. Tsuji et al. (28) have recently reported that α-Gal epitopes are present on human tissue plasminogen activator obtained from blood vessels and from placenta.

Therefore, one may hypothesize that the α1,3GT gene, which is largely suppressed in man (60), may be marginally expressed in man. This might account for the minimal amounts of α-Gal epitopes that are produced in certain human cells. Under physiologic conditions, these epitopes would not interact with anti-Gal in a way that results in a pathologic process. Alternatively, it may be possible that some glycosyltransferases within the thyroid cells have less restricted acceptor and substrate specificity so that a low number of α-galactosyl epitopes could be added to N-acetyllactsosaminyl core epitopes in the Golgi apparatus (100, 101).

The previous findings of Etienne-Decerf et al. (21, 69) on the elevated titers of anti-Gal in the blood of patients with Graves'

disease suggest that aberrant exposure of α-Gal epitopes on thyroid cells to the immune system results in expansion of anti-Gal clones in this disease, as well as the expansion of clones against thyroid-specific antigens. This increase in anti-Gal titer may further suggest that, in these patients, the exposed α-Gal epitopes also may act as an immunogen in addition to the gastrointestinal bacteria which seem to provide the physiologic antigenic stimulation for anti-Gal production (30). A similar mechanism of the exposure of autologous α-Gal epitopes acting as an immunogen was suggested to occur in patients with hepatocarcinoma (106). In these patients, the marked increase in anti-Gal activity subsequent to transcatheter arterial embolization was attributed to cryptic α-Gal epitopes which are exposed by the necrosis of hepatocarcinoma cells induced by the embolization (106).

An alternative cause for increased anti-Gal production might be elevated activity of the gene for α1,3GT, which would result in increased synthesis of α-Gal epitopes on the thyroid cells. The finding of elevated α1,3GT activity in some of the Graves' disease thyroid specimens leads to the speculation that such a process could occur *in vivo*. However, the observed difference in α1,3GT activity between normal and autoimmune thyroid cells is insufficiently significant to directly attribute all autoimmune phenomena to aberrant activity of this enzyme.

It has been shown previously that in murine cells, α1,3GT activity and the expression of its biosynthetic product, the α-Gal epitope, may vary as a result of exposure to retinoic acid, (44) transformation with oncogenes (43), or differentiation from monocytes into macrophages (88). It thus may be possible that a

transient variation in α1,3GT activity which occurred prior to the time of thyroidectomy could have resulted in increased expression of α-Gal epitopes in Graves' patients, and subsequent elevation of anti-Gal titers (21, 69). Hypothetically, the interaction of anti-Gal with α-Gal epitopes on these thyroids might generate a primary lesion, which would lead to the clinical manifestations of Graves' disease.

The present study implies that, if the theory of aberrant α1,3GT activity as a cause for autoimmunity (70) is correct, it requires substantiation by means other than measuring the α-Gal epitope and α1,3GT activity. One possible approach to address this question is the assessment of α1,3GT gene expression in human cells. Whereas DNA sequence homologous to the bovine and murine α1,3GT gene has been found within the human genome (60, 62), no expression of α1,3GT mRNA has been detected in Old World monkey and human cells (60). By using the highly sensitive molecular biology approach of the polymerase chain reaction (PCR), it may be possible to assess whether there is increased expression of this gene in autoimmune human thyroid cells. A distinct correlation between an elevated expression of the α1,3GT gene in thyroid specimens and ongoing autoimmune processes in such tissues would suggest that aberrant activation of the α1,3GT gene can contribute to autoimmunity in man.

Chapter VII

Summary and Perspectives

The study of α-Gal epitopes was of interest to us because preliminary studies indirectly indicated that these epitopes may be expressed in low amounts in humans. This is particularly interesting because approximately 1% of circulating human IgG interacts with the α-Gal epitope. An objective of this dissertation project was to establish the pattern of *in vivo* expression of α-Gal epitopes and their biosynthesis in various mammals. The results of these experiments indicate that these epitopes are normally expressed in large amounts on mammalian glycoproteins. Furthermore, these results support the observation, previously made with cell lines and red blood cells (34), that there was an evolutionary suppression of the synthesis of α-Gal epitopes in man and Old World primates.

Another objective of this dissertation was to develop a sensitive quantitative technique for the detection of α-Gal epitopes on cells. Because it appeared that the synthesis of these epitopes is largely suppressed in man, the detection of these epitopes in human cells and tissues required the development of a highly sensitive assay. The anti-Gal based RIA, developed for the detection of α-Gal epitopes on secreted glycoproteins, was adapted for use with fixed cells and tissues. This enabled the quantitation of these epitopes on transformed human cell lines. Several human cell lines were found which bound anti-Gal at levels 200 to 500 fold less than cell lines from nonprimate mammals. Human cell lines expressing α-Gal

epitopes could also be killed *in vitro* by an anti-Gal mediated ADCC reaction. Thus it was established that transformed human cell lines may express these epitopes at a level which may be physiologically significant.

A third objective of this dissertation was to analyze the possible aberrant expression of α-Gal epitopes on human cells *in vivo*. Since human cell lines were found to express significant levels of α-Gal epitopes, we wondered whether these epitopes were also expressed *in vivo* in human tissues. There was preliminary information by other investigators that the α-Gal epitopes might be present in a cryptic form on normal human thyroids cells (21) and on human red blood cells (66). However, this issue was not clearly established. One means of determining the potential for the expression of these structures in human cells *in vivo* is by directly measuring the enzymatic activity responsible for their synthesis. Therefore, an objective of this dissertation, in conjunction with structural studies, was to develop a sensitive means for the assessment of α-galactosyltransferase (α-GT) enzymatic activity in human tissues. The human thyroid was chosen as the model tissue for the estimation of α-Gal epitope expression and its enzymatic synthesis. The sensitive anti-Gal based RIA was used to determine the amounts of α-Gal epitopes on thyroid cell membranes from normal individuals and patients with Graves' disease. The surprising result of these studies is that these epitopes were found on most of the 11 normal thyroid membranes in addition to those of the 9 Graves' disease thyroids examined. The level of expression of these epitopes was approximately 200 fold less than that observed with

bovine thyroid cells. However, no significant difference between the level of α-Gal epitope expression in the normal or Graves' disease thyroids was detected. Therefore, it was also surprising to find that when α-1,3GT activity was determined in normal and Graves' disease thyroids, three out of the nine Graves' disease thyroid microsome preparations had significantly elevated activities compared with normal thyroid microsomes. Why α1,3GT activity is elevated in some thyroids of individuals with Graves' disease but the density of its enzymatic product in these tissues is not elevated can only be speculated upon. The studies of a variety of different mammalian thyroid cells in this dissertation seem to suggest that competing glycosyltransferase activities are also important in determining the α-Gal epitope expression on cells. One might also speculate that the increase in α1,3GT activity in Graves' thyroids may result in a transient increase in α-Gal epitopes on cells. This might be sufficient for creating a primary lesion mediated by anti-Gal. The ensuing autoimmune reaction might lead to secondary immune stimulation by exposed TSH receptors and thyroglobulin. Thus, by the time the tissue is identified as Graves' disease tissue the α1,3GT enzyme might still be active but the α-Gal epitope need not be synthesized to bring about the clinical manifestations of the disease. It is also worth remembering that anti-Gal titers are significantly elevated in Graves' disease patients (21, 69). This phenomenon might be a result of such an increased α1,3GT activity in Graves' disease tissue, increasing the density of α-Gal epitopes on thyroid cells, perhaps only transiently, and subsequent increased immunization for anti-Gal production.

Since normal thyroid cells in humans appeared to express α-Gal epitopes it was of interest to determine whether this epitope could also normally be expressed in other human tissues. The results of anti-Gal RIA studies with fixed membranes from normal human kidney and liver cells indicate that α-Gal epitopes are present in these tissues. While only two individuals were examined, it is interesting that the level of expression of these epitopes in both normal individuals studied is similar to that observed in human thyroid tissues. One might therefore speculate that expression of these epitopes in some human tissues in very low numbers is the norm. Furthermore, α-GT enzymatic activity could also be detected in liver and kidney cells. This activity was within the same order of magnitude in all human tissues examined but was marginal when compared with the activity measured in bovine tissue microsomes.

In addition to establishing the *in vivo* pattern of expression of α-Gal epitopes, these studies pose new questions regarding the role of these epitopes in human autoimmunity. Since α-Gal epitopes may be present in small amounts in normal human tissues it is not yet clear why these epitopes do not lead to anti-Gal mediated pathological damage. One might speculate that, normally, the α-Gal epitopes are dispersed on cells in a manner in which their density is insufficient to mediate ADCC or complement lysis. Pathological conditions would result when the the threshold density of these epitopes for generation of an autoimmune response would be exceeded. This might be accomplished by an increase in α1,3GT activity in cells, thus, increasing the density of cell surface α-Gal epitopes. This might also be accomplished by an increase in the

clustering of α-Gal epitopes on cells, creating a sufficient local density on the cell surface to trigger an autoimmune response. Exposure of cryptic α-Gal epitopes on cells might also contribute to autoimmunity. Galili suggested that exposure of cryptic α-Gal epitopes on senescent and pathological human red blood cells may be contribute to their destruction (63). Treatment of normal human thyroid was also shown to reveal cryptic α-Gal epitopes (21). Thus, it is possible that the α-Gal epitopes detected in normal tissues in this dissertation are cryptic when expressed in the intact tissue. These epitopes might be exposed in the process of the preparation of membranes from these tissues.

An alternative possibility exists that these α-Gal epitopes are expressed on some cells within tissues in sufficient quantities to trigger an autoimmune reaction. Normally, these cells may be part of a privileged compartment which is sequestered from the humoral immune system, such as the thyroid follicles and parenchymal cells of other tissues. Pathological conditions might result when a breakage of this compartment is made, allowing for contact with the humoral immune system. Therefore, circulating anti-Gal could interact with these epitopes and potentially mediate the destruction of such cells. Evidence which supports this hypothesis was provided by the observation of a marked increase in anti-Gal activity subsequent to transcatheter arterial embolization. This increase in anti-Gal activity was attributed to α-Gal epitopes which are exposed by the necrosis of hepatocarcinoma cells induced by the embolization (106). Other studies have correlated renal injuries and autoimmune diseases associated with the human kidney with an increase in anti-

Gal titers (68, 69). Thus, tissue injury may be one way of increasing the accessibility of α-Gal epitopes for interaction with anti-Gal circulating anti-Gal.

The studies in this dissertation support the notion that, along the evolution of ancestral Old World primates, there occurred a very strong selective evolutionary event, which led to the evolution of primates which could suppress autologous α-Gal epitope synthesis and produce large amounts of antibodies against this structure. Nevertheless, α-Gal epitope suppression in man has not been an absolute one. By a mechanism which is not yet clear, human cells produce low amounts of the α-Gal epitope *in vivo* and *in vitro*, aberrant expression of these epitopes on malignant cells may lead to an anti-Gal mediated destruction of such cells.

There is still no direct evidence for the consequences of the interaction of anti-Gal with α-Gal epitopes on human cells *in vivo*. One study addressed this issue, using the mouse as a model (110). This study suggested that the interaction of anti-Gal with α-Gal epitopes on the cell surface could mediate killing of the such cells by cells of the murine reticuloendothelial system *in vivo*. Even though the number of α-Gal epitopes on human cells expressing aberrantly these epitopes appears to be much less than that of murine cells, the studies in this dissertation raise the possibility that this number is sufficient for anti-Gal mediated killing *in vivo*. Such interactions with anti-Gal *in vivo* might initiate an autoimmune reaction.

The evaluation of the relationship between α-Gal epitope expression *in vivo* and autoimmune diseases awaits future studies at the molecular level. The basis of the aberrant expression of the

α-Gal epitope on transformed human cell lines will first be investigated. The tools are currently available to address the hypothesis that the expression of these epitopes on human cells is due to the activation of the human α1,3GT gene, which may be highly homologous with the bovine (60) and murine (62) α1,3GT genes. Studies in this dissertation suggest that this gene may normally be expressed at low levels in some human cells. It could be speculated that the expression of this gene might be enhanced by factors such as malignant transformation, viral, or chemical mutagenesis. It is also possible that this aberrant α1,3GT activity is not due to a homologue of the characterized mammalian enzyme but is due to the activity of a "promiscuous" glycosyltransferase. This activity might be capable of adding terminal α-Gal structures onto Galβ1-4GlcNAc-R acceptors to synthesize the α-Gal epitope. Probing human cells with cDNA constructs capable of hybridizing with α1,3GT mRNA might allow the distinction to be made between activity due to a different "promiscuous" glycosyltransferase and the authentic α1,3GT. Incorporation of polymerase chain reaction methodology to these hybridization assays might allow marginal transcription of α1,3GT mRNA to be detected in human cells. This methodology might further be applied to the study of human normal and autoimmune tissue samples. Graves' disease could be used as a model autoimmune tissue for these studies, since preliminary studies in this dissertation have suggested the presence of α1,3GT activity in cells of the thyroid.

Perhaps the most conclusive way of examining the expression of α-Gal epitopes in human tissues would be by the direct isolation

and sequencing of such epitopes. The high affinity of anti-Gal for for α-Gal epitopes might be utilized to construct an affinity column for glycoconjugates containing such epitopes. However, it is expected that the isolation of α-Gal epitopes from human tissues would be an extremely difficult to accomplish. Preliminary studies in this dissertation have suggested that there may be 500 fold fewer α-Gal epitopes in human tissues compared with other mammals, such as the cow. Therefore, isolation of sufficient quantities of α-Gal epitopes from human tissues for chemical sequence analysis would require very large quantities of such tissues. Such quantities of human tissues were not available to us in the process of this dissertation work. While it may be possible to purify and sequence α-Gal epitopes from human tissues these studies await suitable quantities of such tissues.

The tools developed in this dissertation to study the expression of α-Gal epitopes on normal and pathological human tissues will complement future studies addressing the molecular basis of the expression of these epitopes. Further studies will focus on studying the molecular basis for the suppression of the α1,3GT gene in human cells. This will include an analysis of the structure of the human α1,3GT gene homologue. Structural analysis of this gene is important because it remains an open question if the suppression of this gene in humans is due to a regulatory mutation, possibly in the 5' untranslated region, or is due to mutations in the structural gene. Once these issues are addressed, then the role of the molecular basis of α1,3GT activity in normal and pathological tissue specimens will be examined. This will allow questions to be addressed regarding

the potential for aberrant α1,3GT gene expression in human autoimmune diseases, malignancies, and other pathological processes.

Since anti-Gal is present in such large amounts in humans it is interesting to note that the levels of this antibody were found to increase 2 to 8-fold in various diseases, such as Graves' disease, Henoch-Schonlein purpura, IgA nephropathy, and rheumatoid arthritis (21, 67, 68, 69, 97). It is thought that the generation of an antibody mediated immune response involves the activation and proliferation of circulating memory cells capable of recognizing a particular antigen. Therefore, if anti-Gal elevations are due to the expansion of B cell clones capable of recognizing α-Gal epitopes, one would expect to find such clones circulating in the blood. With this idea in mind, experiments were designed, as described in the Appendix, to isolate and analyze the circulating B cells for anti-Gal secretion. It was found that the number of anti-Gal secreters as a percentage of the total proliferating cells closely matched the percentage of the total circulating antibodies represented by anti-Gal. The tools developed in these preliminary studies will provide a valuable basis for future studies attempting to correlate the elevation in anti-Gal titers with proliferation of anti-Gal secreting B-cells.

APPENDIX

Analysis of Anti-Gal Production by Circulating B Lymphocytes

Introduction

Anti-Gal comprises as much as 1% of circulating IgG in humans (16, 27). While the presence of circulating anti-Gal is a well characterized phenomenon, little is known about the B lymphocytes (HBL) which secrete these antibodies. Hypothetically, it is possible that approximately 1% of B lymphocytes are capable of producing anti-Gal, since this antibody constitutes such a large proportion of circulating immunoglobulins in man. The well accepted theory of clonal diversity in B lymphocytes states that there can be a potential production of 10^8 different antibody specificities (117). Therefore, the possible commitment of 1% of B lymphocytes to the production of antibodies with a particular specificity would be exceptional. It was therefore of interest to determine the proportion of B lymphocytes which are capable of producing anti-Gal. It was suggested by Galili et al. (30) that anti-Gal secretion results from antigenic stimulation by gastrointestinal bacteria expressing the α-Gal epitopes. Studies by other investigators demonstrated that circulating HBL could be isolated and immortalized by infection with the Epstein-Barr virus (EBV) (112). These EBV transformed cells have been found to be capable of secreting antibodies against defined antigens, such as human thyroglobulin (113), tetanus toxoid (113), and rhesus D blood group antigen (114). By immortalizing human blood B lymphocytes, using EBV, we determined the proportion of such lymphocytes

producing anti-Gal. We could show that approximately 1% of B lymphocytes produce this natural antibody.

Results

As described in Materials and Methods, 40,000 normal HBL were transformed in microtiter wells by infection with EBV, followed by a period of 2-3 weeks, in which cells were allowed to proliferate in the microtiter wells. The supernatant from these wells was removed and agglutination assays were performed by incubating rabbit red blood cells (rrbc) with these supernatants. Since rrbc have large numbers of α-Gal epitopes (23), anti-Gal in these supernatants will bind to these rrbc. High titer or polyvalent antibodies may bridge between epitopes on different rrbc, causing agglutination of these cells. Alternatively, anti-Gal antibodies may bind to rrbc but lack the ability to cause a direct agglutination. Therefore, incubation with supernatants was followed by an incubation of the cells with anti-human Ig, as a secondary antibody. As is shown in Table VI, the percentage of microtiter wells with proliferating HBL secreting anti-rrbc antibodies was between 3.9% and &.7.7%, with an average of 5.6%. These antibodies were present in culture supernatants of transformed HBL from all 13 individuals tested, who were of different ABO blood types.

As a control for the ability of transformed HBL to secrete anti-Gal, it was of interest to determine whether transformed HBL were capable of secreting other natural antibodies. Two well characterized natural antibody activities are the anti-blood group A and anti-blood group B antibodies. The ability of transformed HBL to secrete

antibodies recognizing these structures was assessed with aliquots of the same supernatants used for screening for anti-Gal production, by agglutination of blood group A or B human red blood cells (hrbc) (Table VI). Autologous type A or B hrbc were used for agglutination assays. As is seen in Table VI, all donors tested had transformed HBL which produced antibodies capable of agglutinating hrbc of a nonself blood type but could not agglutinate autologous rbc. These results follow the same pattern as is seen with anti-blood group antibody activity in human serum (72). The percentages of wells with anti-A or anti-B activity were 3 to 7-fold less than those which secreted anti-rrbc activity.

Table VI

Production of anti-Gal by EBV Transformed B Cells

Donor[a] Number	Blood Type	Percent [b] anti-Gal	Percent* anti-A	Percent* anti-B
1	O	4.5	ND	ND
2	O	4.5	ND	ND
3	O	7.0	1.0	1.8
4	O	7.7	1.5	2.1
5	A	5.7	0.0	0.5
6	A	4.0	0.0	ND
7	A	5.2	0.0	1.4
8	A	7.5	0.0	1.9
9	A	5.0	0.0	1.3
10	A	5.4	0.0	2.1
11	A	3.9	0.0	1.3
12	B	7.0	1.3	0.0
13	B	6.0	1.5	0.0

[a] B cells from normal donors were transformed by Epstein-Barr virus and cultured for 2-3 weeks in microtiter wells prior to assay.

[b] Values are expressed as the percentage of microtiter wells with the ability to agglutinate rabbit erythrocytes. The number of wells which were examined was 260 to 384 per assay.

* Values represent the percent of microtiter wells agglutinating blood group A rbc or B rbc, respectively. When individuals were examined for anti-self blood group antigen reactivity, autologous rbc were used.

ND = not done

It was of interest to determine the number of proliferating HBL per well in order to assess what percentage of antibody secreting HBL clones are capable of secreting anti-Gal. The strategy we used to address this question was to expand wells containing anti-Gal secreting transformed HBL to 10^7 cells. DNA was isolated from these cells, treated with restriction endonucleases and Southern blot analysis with a probe to the 5' invariant region of immunoglobulin genes was performed. Restriction fragments are of different lengths due to B lymphocyte clones with different types of somatic rearrangement of the Ig genes. Therefore, the number of bands seen in each lane of the blot is an indication of the number of the major antibody producing clones per well. The number of clones ranged from 2 to 6 with an average of 5 per well (not shown). Since around 384 wells with proliferating transformed HBL were examined per individual, this represented a total of 768 to 1920 total HBL clones. Because the average frequency of anti-Gal producing wells was 5.6% of the total wells, it is likely that the anti-Gal in supernatants from each of these wells is the product of single transformed HBL clones. Therefore, this suggests that the average percentage of proliferating transformed HBL clones capable of secreting anti-Gal is approximately 1.1%.

While it was likely that the rrbc agglutinating activity seen in HBL culture supernatants was anti-Gal, this assumption required confirmation. The specificity of agglutination of rrbc was investigated by adsorbtion of agglutinating activity on synthetic carbohydrates linked to Synsorb beads. As described in the footnotes to Table VII, HBL culture supernatants were passed through either Synsorb-115 (with synthetic Galα1→3Galβ1→4GlcNAc structures) or Synsorb-LacNAc (with synthetic Galβ1→GlcNAc structures). After extensive washing, columns were eluted at low pH. Eluates were neutralized and tested for agglutination of rrbc. The results presented in Table VII show that rrbc agglutinating activity could be eluted from Synsorb-115 but not from Synsorb-LacNAc columns. These results are similar to those obtained by studies of the specificity of anti-Gal from human serum (28). In supernatants from all clones, except one, passage of 15ml of these supernatants through Synsorb-115 columns and elution of these columns in a 1ml volume resulted in a concentration of agglutinating activity, as seen by an increase in the agglutination titer. The reason that the level of increase in the concentration of agglutinating activity varied from one clone to another might be due to a difference in affinities of the different anti-Gal clones for the Galα1→3Galβ1→4GlcNAc structures linked to Synsorb beads. Thus, during the washing of the Synsorb-115 columns, antibodies bound to these structures through relatively low affinity interactions might have had a greater tendency to be washed from the columns. Since these agglutinating activities probably represent different anti-Gal secreting clones, it is not surprising that the antibodies produced by these cells exhibit

different affinities for α-Gal epitopes. This study suggests that the interaction of these antibodies with Galα1→3Galβ1→4GlcNAc structures is specific, since the antibodies did not interact with carbohydrate structures lacking the terminal Galα1→3Gal linkage.

Table VII

Specificity of Rabbit Erythrocyte Agglutinating Activity

Clone[a] Number	Original titer	Syn-115[b] Eluate Titer	Syn-LacNAc[c] Eluate Titer
1	1:1	1:2	0
2	1:4	1:32	0
3	1:2	1:4	0
4	1:8	1:8	0
5	1:2	1:8	0
6	1:16	1:3	0
7	1:8	1:32	0
8	1:2	1:8	0
9	1:2	1:16	0
10	1:2	1:8	0
11	0	1:1	0
12	1:1	1:4	0

[a] EBV transformed B lymphocytes, which produced antibodies capable of agglutinating rrbc were expanded from microtiter wells to flasks. Supernatants were removed from flasks and used for this assay.

[b] Culture supernatants in volumes of 15 ml were passed through minicolumns containing 1ml of Synsorb 115 beads, a matrix with synthetic Galα1$\rightarrow$3Galβ1$\rightarrow$4GlcNac-R structures. After washing with 15 ml of PBS, columns were eluted with 1ml of 0.2M glycine-HCl and immediately neutralized with NaOH. Serial dilutions of eluates were made and Rabbit erythrocyte agglutination titers were then assessed.

[c] Supernatants were treated in the same fashion as those passed through the Synsorb 115 columns. Synsorb LacNAc contains synthetic Galβ1$\rightarrow$4GlcNac-R structures.

The anti-Gal reactivity of these clones could be further demonstrated by their binding to bovine BS^+ thyroglobulin, which, as discussed in Chapter III, expresses an abundance of α-Gal epitopes. In these studies, an ELISA (enzyme-linked immunosorbant assay) was performed by incubating anti-Gal, affinity purified on Synsorb-115, from seven different transformed HBL clones, which secreted antibodies capable of agglutinating rrbc, with BS^+ thyroglobulin coated microtiter wells (microtiter wells were coated as described for RIA assays in Materials and Methods), followed by incubation with secondary anti-human antibodies directed against IgG, IgM or IgA conjugated to horseradish peroxidase. The data from these assays are shown in the form of OD at 450nm, as an indication of binding activity in the supernatants (Figure 24). It was also of interest to compare the binding activity of these monoclonal antibodies with the polyclonal anti-Gal, isolated from human plasma. The binding of 2μg/ml affinity purified anti-Gal (as described in Material and Methods) from plasma to BS^+ thyroglobulin was assayed in parallel with the culture supernatants. As shown in Figure 24, all clones bound to a varying degree to the thyroglobulin. The monoclonal anti-Gal affinity purified from supernatants 1 through 5 was was of the IgG class. As would be expected, the anti-Gal from plasma was mostly IgG. However, lower amounts of IgM and IgA antibody activity was also detected. This is in accord with studies which suggested that anti-Gal from plasma is composed of all Ig classes, but is mostly IgG (72). It was also interesting to note that while monoclonals 6 and 7 could agglutinate rrbc, their interaction with BS^+

thyroglobulin was very low. As discussed below, these two clones display a high affinity to glycolipids with the α-Gal epitope.

Murine laminin was another solid phase antigen used for studying anti-Gal activity of these monoclonal antibodies. As mentioned in the introduction to this dissertation, murine laminin contains a large number of α-Gal epitopes (9). In accordabce with the results presented in Figure 25, the binding of monoclonal anti-Gal purified from supernatants 1 through 4 was seen with murine laminin as well in the ELISA.

We also studied the binding of these monoclonal antibodies to glycolipids. The major glycolipid on rrbc is ceramide pentahexoside (Galα1→3Galβ→4GlcNAcβ1→3Galβ1-4Glc1-1ceramide; CPH), containing terminal α-Gal epitopes (22). Supernatants were added to microtiter wells which were coated with either CPH or Galβ→4GlcNAcβ1→3Galβ1-4Glc1-1ceramide (paragloboside) as solid phase antigens. Whereas monoclonal anti-Gal numbers 6 and 7 could not interact with α-Gal epitopes on the glycoproteins, thyroglobulin and laminin, they strongly interacted with CPH (Figure 26). These monoclonal anti-Gal antibodies were of the IgM class. Only two of the other five monoclonals interacted with CPH. Anti-Gal Ig from plasma bound to CPH as was expected. The specificity of binding of these antibodies to CPH was demonstrated by the inability of these antibodies to interact with another defined glycolipid, paragloboside, which lacks the terminal Galα1→3Gal structure (Figure 26).

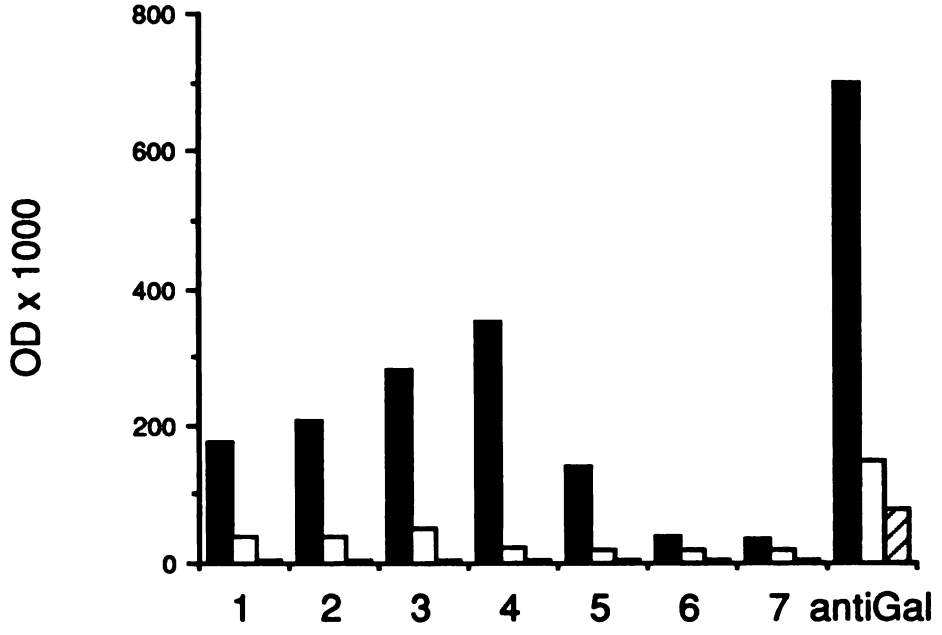


Figure 24. Interaction of antibodies secreted by EBV transformed B lymphocytes with BS+ thyroglobulin in an ELISA. Filled bars represent IgG, while open bars represent IgM. No IgA reactivity was found. Microtiter wells were coated with BS+ thyroglobulin, as described in Materials and Methods. Wells were then blocked with 1% BSA. Monoclonal antibodies affinity purified on Synsorb-115 from supernatants of seven different EBV transformed B lymphocyte cell lines were added to microtiter wells coated with BS+ thyroglobulin. Following incubation of these monoclonal preparations or 2μg/ml anti-Gal purified from AB plasma overnight at room temperature, wells were washed and incubated with mouse anti-human IgG, IgM or IgA (conjugated to horseradish peroxidase). Optical density (OD) measurements were made at 450nm, after incubation with peroxidase substrate for 1 hour. Bars represent OD values for the interaction with BS+ thyroglobulin minus nonspecific OD values (binding to BSA coated wells).

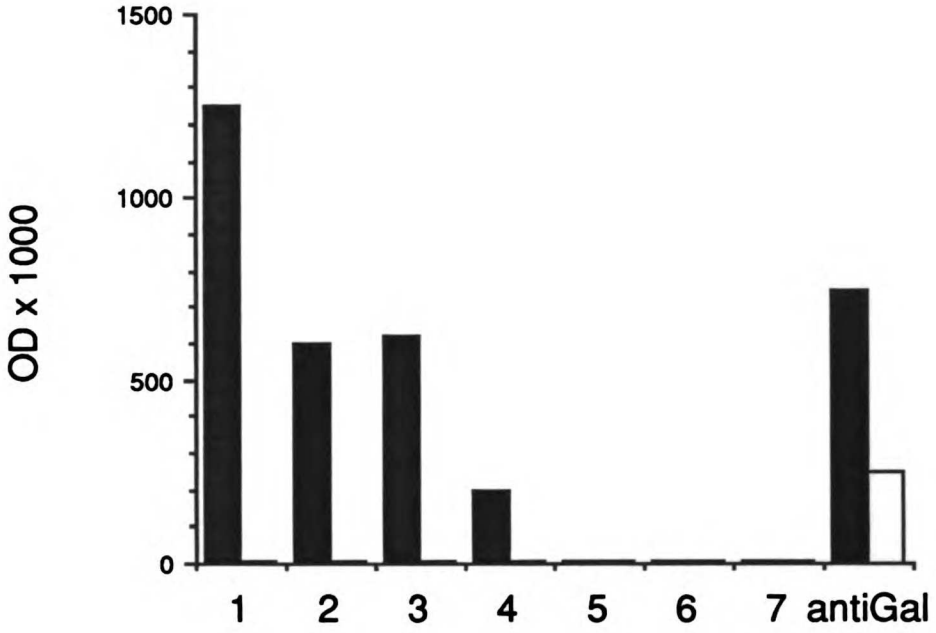


Figure 25. Interaction of antibodies secreted by EBV transformed B lymphocytes with murine laminin in an ELISA. The open bar represents anti-Gal IgM. Affinity purified antibodies from supernatants from seven different EBV transformed B lymphocyte cell lines or affinity purified anti-Gal from AB plasma were added to microtiter wells coated with murine laminin. ELISA's were carried out with the same supernatants as described in Figure 26. Bars represent OD values for the interaction with murine laminin minus nonspecific OD values for binding to BSA. No specific interaction of these supernatants was seen with human laminin as the solid phase antigen (not shown).

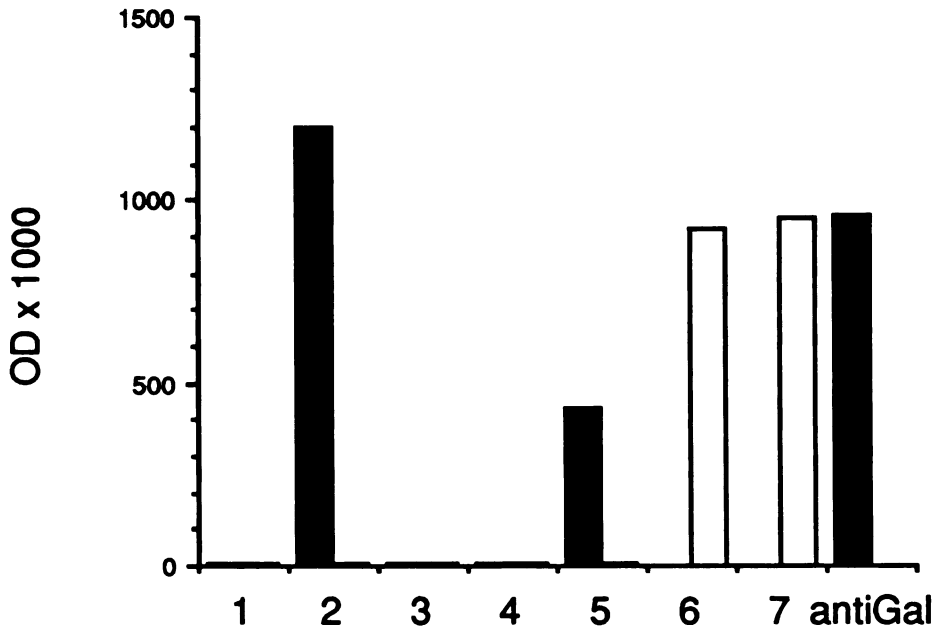


Figure 26 Interaction of antibodies secreted by EBV transformed B lymphocytes with ceramide pentahexoside (CPH) in an ELISA. Open bars represent monoclonals which interacted with CPH but not with the glycoproteins in the assays of Figures 26 and 27. ELISA's were carried out with the same monoclonal antibodies purified from supernatants of as described in Figures 26 and 27. Nine nmol CPH in ethanol was added to wells and dried down. After blocking wells with BSA, affinity purified monoclonal antibodies or anti-Gal affinity purified from plasma were added to CPH coated microtiter wells. Bars represent OD values for the interaction with CPH minus nonspecific ODs for the interaction with BSA. No specific interaction of antibodies in these supernatants was seen with paragloboside as the solid phase antigen.

Discussion

Since anti-Gal is such an abundant natural antibody in humans, we wondered whether a large proportion of circulating HBL were also capable of secreting this polyclonal antibody. Therefore, the first objective of these studies was to develop a technique for determining the secretion of anti-Gal by circulating HBL. Studies by other investigators indicated that EBV transformation and immortalization of HBL, *in vitro*, could produce cell lines capable of secreting antibodies (112, 115). The efficiency of transformation of HBL obtained by us was approximately 1 in 10^4 HBL, which was also observed by Taub, et al. (116). However, the number of proliferating HBL clones obtained by this method does not correlate in a linear fashion with the number of HBL per microtiter well. Thus, DNA analysis of the number of proliferating HBL clones obtained in these studies indicated approximately 10 clones could be obtained when 40,000 HBL were infected with EBV.

By EBV transformation and growth of HBL in microtiter wells, we were able to isolate what are probably monoclonal anti-Gal secreting HBL clones. The agglutination of rrbc with supernatants from microtiter wells provided a rapid method for the preliminary screening of HBL capable of secreting anti-Gal. Cells from wells which could secrete rrbc agglutinating antibodies were expanded and selected for those clones which could continue to produce anti-rrbc antibodies. The specificity of the monoclonal antibodies secreted by these cells was demonstrated as anti-Gal by adsorbtion on affinity matrices containing synthetic α-Gal epitopes but not on a matrix

containing carbohydrates lacking these epitopes. These monoclonal anti-Gal antibodies also did not interact with blood group antigens on human red blood cells.

The results of the studies of the interaction of these monoclonal anti-Gal antibodies with glycoproteins containing α-Gal epitopes suggest that not all of these antibodies can interact with glycoproteins. Only one monoclonal antibody out of seven tested could interact strongly with both α-Gal epitopes on CPH, which was dried onto microtiter wells, and α-Gal epitopes on glycoproteins. Two monoclonal anti-Gal antibodies were found which could interact readily with α-Gal epitopes on glycolipids but only weakly with glycoproteins. This lack of ability to bind to α-Gal epitopes on glycoproteins was also observed with the mouse monoclonal antibody, Gal-13 (48).

Whereas the two antibodies which could only interact with α-Gal epitopes on glycolipids were of the IgM class, 15 different monclonal anti-Gal antibodies which could interact with α-Gal epitopes on glycoproteins were of the IgG class and only one of the IgM class. Thus 3 HBL clones out of 18 examined secreted anti-Gal IgM and no clones were found which could secrete anti-Gal IgA. The Ig class distribution seems to suggest that the *in vitro* production of anti-Gal by transformed HBL reflects the ability to produce such antibodies *in vivo*. Antibodies capable of agglutinating rbc of another blood group were observed in all individuals tested. However, antibodies capable of interacting with autologous rbc were not detected. This phenomenon, associated with the mechanisms of immunological tolerance, has also been observed with circulating

antibodies directed against ABO blood groups (72). The percentage of clones secreting anti-A or anti-B rbc antibodies was much lower than the percentage of clones secreting anti-Gal.

These studies demonstrate the isolation of circulating HBL which are capable of secreting anti-Gal. These studies also establish a value for the average percent of circulating HBL in normal individuals which can secrete anti-Gal (1.1%) and the class of anti-Gal antibodies secreted (approx. 85% IgG, 15% IgM) by these HBL. These preliminary studies establish a basis for future research dealing with the relationship between elevated anti-Gal titers, as seen with Graves' disease (21, 69) or Henoch-Schonlein purpura (68), and the clonal proliferation of circulating HBL, capable of secreting anti-Gal.

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