THY-1 GLYCOLIPID:
RADIOLABELING AND ISOLATION FROM
BRAIN AND LYMPHOMA CELL LINES

by

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A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1979

ABSTRACT

THY-1 GLYCOLIPID: RADIOLABELING AND
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Mouse Thy-1 glycolipids were isolated and characterized by radiolabeling, thin layer chromatography (TLC) and an immune response plaque forming cell (PFC) assay. Brain gangliosides were labeled by intracranial injection with [1-^{14}C]N-acetylmannosamine (ManNAc), extracted, separated by one and two dimensional TLC, and visualized with autoradiography. The gangliosides were assayed for Thy-1 activity with the anti-Thy-1 PFC assay. One Thy-1 active compound demonstrating Thy-1 alloantigenic specificity was identified from either Thy-1.1 or Thy-1.2 strains.

Lymphoma cell lines were incubated with either [1-^{14}C]palmitate or [1-^{14}C]ManNAc, extracted, separated by two dimensional TLC and visualized by autoradiography. Anti-Thy-1 PFC assay identified one Thy-1 glycolipid from either BW5147 cells (Thy-1.1) or S49.1 cells (Thy-1.2) which demonstrated alloantigenic specificity.

Neuraminidase and mild HCl treatment of Thy-1 glycolipids abrogated the subsequent PFC response. Thy-1 bound to DEAE cellulose as expected for gangliosides. Thy-1 antigenicity is associated with a ganglioside in mouse brain and lymphoma cells.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Walter Esselman for his support and assistance during my studies. For their technical aid and advise, I also thank Tang Jang Wang, Warren Coon, and Joyce Wildenthal. In addition, this report would not have been what it is without the grammatical guidance of Drs. Tobi Jones and Myra Jennings. The excellent typing of Debbie Densmore is greatly appreciated. Finally, thanks to my parents, friends, and Nanc who managed to keep me sane.

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ABBREVIATIONS

Galactose, Gal; Glucose, Glc; Fucose, Fuc; Mannose, Man; GlcNac, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid; asn, asparagine; ser, serine; thr, threonine; UDP, uridine diphosphate; GDP, guanosine diphosphate; CMP, cytosine diphophate.

REVIEW OF THE LITERATURE

Glycoconjugates are a diversified group of substances comprised of glycolipids, glycoproteins and proteoglycans which have a carbohydrate substituent covalently bound to a protein or lipid core. These substances are generally found in cell membranes or body fluids and secretions. Various monosaccharide structures comprise the carbohydrate component which may vary in size from one to more than a hundred sugars in different branching configurations. The complex carbohydrates appear to be a confusing group but classifications can be made based on the composition and sharing of oligosaccharide components. For example, the glycolipids were recently subdivided by Sweeley and Siddiqui (1) into seven families based on the composition of the tetrasaccharide cores. Proteoglycans and glycoproteins are classfied by the composition of their oligosaccharide cores (2,3,4,5) as well as the nature of their glycopeptide linkages.

The presence of common carbohydrate components on glycoconjugates is a reflection of common metabolic pathways shared by members of these families. It is important to note that glycoconjugates with similar carbohydrate structures may be functionally and immunologically related. The intent of this review is to present an introduction to basic glycoconjugate concepts and then look at a specific example in detail. Thy-1 cell surface antigen is a subject which lends itself well to this discussion. Evidence supporting several chemical states such as glycolipid and glycoprotein, has been presented for Thy-1 antigen.

I. Introduction to Glycoconjugates.

The Glycosphingolipids. Compounds which contain ceramide (N-acyl sphingosine) as their lipid component covalently linked to one or more

carbohydrates are referred to as glycosphingolipids. These glycolipids are present in all cell membranes with an array of carbohydrate components. Their orientation is such that the hydrophobic tail (ceramide) is dissolved in the fluid membrane and the hydrophillic head (oligosaccharide) extends into the extracellular aqueous environment (6). The oligosaccharide moiety is made available for receptor functions, surface interactions with other cells and antigen antibody reactions by this positioning. Two basic groups of glycosphingolipids are determined by the presence or absence of N-acetylneuraminic acid (sialic acid, NeuAc) in the oligosaccharide (1). Those which lack sialic acid are called neutral glycolipids and those of which sialic acid is a part are called gangliosides.

The biosynthesis of ceramide is basically a condensation between the fatty acid palmitate and the amino acid serine (7,8,9). This combination invloves several enzymatic steps resulting in the single long chain base, sphingosine: $CH_3-(CH_2)_{12}-CH=CH-CH(OH)-CH(NH_2)-CH_2OH$. In all glycosphingolipids, the amino group of sphingosine participates in an N-acyl linkage with a free fatty acid producing a double long chain compound named ceramide. Gangliosides usually contain steric acid [$CH_3-(CH_2)_{16}-COOH$] as the N-acyl group.

Glycolipid biosynthesis is believed to occur in the Golgi apparatus (10,11,12). The initial step involves the transfer of a sugar moiety from the nucleotide carrier of either UDP-Gal or UDP-Glc to the C-1 hydroxyl group of ceramide. The carbohydrate chain is lengthened by sequential addition of monosaccharides from sugar nucleotides (UDP-Gal, UDP-GalNAc, GDP-Fuc, CMP-NeuAc) to the nonreducing end of the oligosaccharide on the nacent glycolipid (10,13). A relatively minor pathway

also exists for the biosynthesis of monoglycosylceramides, commonly known as cerebrosides (14,15).

Biosynthetic radiolabeling of glycolipids is a great aid in studying their normal and abnormal metabolism. This may be done either *in vitro* or *in vivo* by supplying cells with a radiolabeled glycolipid precursor. Palmitate has been used successfully by several investigators to monitor glycolipid metabolism. Robbins and Macpherson (16) radiolabeled NIL-2 cell glycolipids *in vitro* by adding [1-^{14}C]palmitate to the culture media. The rate at which the cells incorporated the palmitate into glycolipids allowed these investigators to monitor the dependency of glycolipid synthesis on cell density. This precursor appears to label all glycosphingolipids (17,18). An *in vivo* procedure which specifically labeled gangliosides was developed by Kolondy et al. (19). Radiolabeled N-acetylmannosamine (ManNAc) was injected intracranially into seven day old rats. Two days were allowed for incorporation of the label and the brains were then removed and the gangliosides were extracted. Chemical analysis of the ganglioside components traced all radioactivity to the sialic acid moieties. These experiments support the theory first proposed by Coombs and Roseman (20) that ManNAc is the direct precursor of NeuAc. The enzymes responsible for this conversion were later identified in mammalian tissue (21). ManNAc is condensed with phosphoenolpyruvate to form a derivative of NeuAc which is dephosphorylated to yield free NeuAc. This acidic sugar is then linked to the nucleotide CMP to give CMP-NeuAc, the form required for oligosaccharide biosynthesis.

The Glycoproteins. Surface membrane glycoproteins mediate many of the interactions of cells with their extracellular environment (22).

The observation that glycoproteins are mobile in the fluid membrane matrix of erythrocyte stroma (23) and culture cells (24) probably holds true for all cells. These surface components are oriented such that hydrophobic amino acids spatially next to the C-terminus end of the protein are dissolved in the membrane bilayer and the various carbohydrate moieties spatially near the N-terminus end are exposed to the external environment (25,26). The carbohydrate substituents have proven to be important in the function of glycoproteins. If the terminal sialic acids are cleaved from serum glycoproteins, they are quickly removed from the circulation by liver parenchymal cells (27). Desialylation of lymphocytes with neuraminidase treatment drastically effects the normal circulatory patterns these cells follow (28).

Characteristic sugars are present in the oligosaccharide moieties of glycoproteins whereas the amino acid compositions are not espically unique. The monosaccharides found in serum and mucin glycoproteins are D-galactose, D-glucose, L-fucose, D-mannose and two amino sugars N-acetylglucosamine and N-acetylgalactosamine. In addition several sialic acids are found in the N-acetyl and N-glycolyl forms (29). The carbohydrate moieties can vary in size from one residue in ribonuclease B (30) to 500 resudues in submaxillary mucins (31).

Two major and one minor type of glycopeptide linkages are found in serum and mucin glycoproteins (32). Mucin glycoproteins, found in epithelial secretions, contain only the GalNAc-serine (or threonine) linkage. This is an O-glycosidic bond between C-1 of GalNAc and the B-hydroxy group of serine or threonine and can be detected by its sensitivity to mild alkali conditions (0.1N NaOH at 37^{o} for 48 hr). The other major type of linkage is an N-glycosidic bond between C-1 of GlcNAc

and the amide group of asparagine. This is the predominant linkage in serum and plasma membrane glycoproteins and it is resistant to mild alkali conditions. A minor linkage type is found only in collagen and basement membrane glycoproteins. It is an O-glycosidic bond between Gal and hydroxylysine and is the most alkali resistant of the three.

The most well known of the mucin glycoproteins are those with blood group activity found in epithelial secretions such as saliva, meconin, and gastric juice (33,34,35). These glycoproteins contain only the O-glycosidic linkage between GalNAc-serine (or threonine) and have "complex" type oligosaccharide chains with very small amounts of mannose and no glucose. Most humans are secretors and have glycoproteins in their epithelial secretions with the same blood group activity as their blood type. Non-secretors have glycoproteins in their epithelial secretions which lack the terminal carbohydrate sequences that determine blood group specificity.

Serum glycoproteins such as fetuin (32), thyroglobulin (36) and immunoglobulin (37) as well as erythrocyte membrane glycoproteins (38) have been found to contain the N-glycosidic linkage between GlcNAc-asparagine as their predominant glycopeptide linkage. All of the above mentioned glycoproteins also contain minute quantities of the GalNAc-serine type linkage. Oligosaccharide chains attached to proteins by the N-glycosidic linkage are referred to as "simple" because they contain large quantities of mannose and no glucose.

More than one biosynthetic pathway exists for the synthesis of glycoproteins. The sequence of events concerning the GlcNAc-asparagine linkage of serum and plasma membrane glycoproteins will be described here. This basic process involves building the core oligosaccharide on

a lipid intermediate, transfer of the core to a specific asparagine residue on a protein, and addition of the terminal carbohydrate sequences to the nascent glycoprotein. This contrasts with the assembly of glycosphingolipids where all sugars are added sequentially to the nascent glycolipid. Another pathway for serine threonine linked glycoproteins not involving a lipid intermediate has been reviewed (39).

Asparagine linked glycoprotein oligosaccharides are synthesized in the cisternae of the rough endoplasmic reticulum and the Golgi apparatus (4,5). The membrane bound glycosyltransferases responsible for oligosaccharide assembly are found here (4,10,13). The initial step is the transfer of GlcNAc from UDP-GlcNAc to a polyisoprenoid phospholipid called dolicol phosphate (3). The chain is lengthened and branched with the addition of GlcNAc and Man from UDP-GlcNAc and GDP-Man to the nonreducing end of the first GlcNAc residue attached to the dolicol phosphate acceptor (3,40). Three glucose residues are added to the nascent core oligosaccharide (5,41) and the core is transfered from the lipid carrier to a specific asparagine residue on the protein (42). This transfer absolutely requires the presence of glucose on the core oligosaccharide (41) and probably a specific amino acid sequence is recognized as the acceptor site of the transfer. The amino acid sequence asn·x·thr(ser) has been shown to be a prerequisite for GlcNAc-asn linkages (43,44). The transfer of the core oligosaccharide may even occur as the protein is synthesized on the ribosomes (45,46). Terminal glucose and some mannose is removed in a processing step and the core oligosaccharide is now complete (47). The glycoprotein now appears in the Golgi apparatus where additional monosaccharides are added to the core oligosaccharides from UDP-Gal, GalNAc, and GlcNAc; GDP-Fuc and Man; and

CMP-NeuAc.

The glycosyltransferases which catalyze the addition of the terminal carbohydrate chains are specific for the sugar nucleotide donor, the oligosaccharide acceptor and the linkage and anomerity of the glycosidic bond formed (5,10,13). The observation that the product of one glycosyltransferase reaction becomes the substrate for the next reaction in the sequence has led some researchers to propose the existance of multienzyme complexes which would synthesize a complete carbohydrate chain (13,48). A glycoprotein's core oligosaccharide would be recognized by a particular complex and become the substrate for the addition of its terminal sequences.

The process of attaching the terminal carbohydrates to glycoproteins in the Golgi apparatus may hold the key to understanding the functional similarities of glycoconjugates. The terminal three or four sugars in the oligosaccharide of glycoproteins are responsible for their receptor functions and antigenic specificity. Blood group specificity is determined by the terminal carbohydrate and its anomerity and linkage to the penultimate residue as is discussed below. Glycolipids and glycoproteins with the same blood group specificity have identical terminal carbohydrate structures (49,50). This in turn, may reflect the sharing of common glycosyltransferase complexes which synthesize the terminal residues.

II. Glycoconjugates Sharing Antigenic Activity

Carbohydrate moieties are important to the biological and immunological activity of glycoconjugates. These moieties are carried on the terminal end of an oligosaccharide covalently attached to either protein or lipid. The carbohydrate substituents are oriented in the membrane so

they are free to interact with the extracellular environment. Proteins and lipids with the same immunological specificity also share identical terminal carbohydrate sequences. This section will briefly review this relationship as it applies to some of the blood group glycoconjugates. In the next section, evidence will be presented supporting the contention that Thy-1 antigenicity is also found on glycoconjugates.

Blood Group P_1. This example illustrates the effects minor structural changes can make in immunological activity. Blood group P_1 glycolipid was first identified by Naiki et al. (51,52). This phenotype is present in 75% of the population (53) and is detectable on erythrocytes (54), fibroblasts and lymphocytes (55). A glycolipid isolated from rabbit erythrocytes, was found to be compositionally identical to human P_1 glycolipid except that blood group B activity was detected instead of P_1 (56,57). The difference in immunological specificity was accounted for by P_1 glycolipid having a terminal Gal (α1-4) linkage but the rabbit glycolipid had a terminal Gal (α1-3) linkage, the same as blood group B substances. This illustrates that the terminal sugar and its anomerity and linkage to the penultimate residue can determine immunological specificity.

Blood Group B. Glycoproteins and glycolipids which have a terminal Gal (α1-3) Gal (α1-3) GlcNAc- trisaccharide display blood group B specificity. Three glycolipids that share this trisaccharide and display B activity have been isolated from human erythrocytes (58,59,60). Two of these are hexaglycosylceramides and differ only in the linkage of the penultimate Gal to the GlcNAc and the other contains an additional fucose residue. A B active megaloglycosphingolipid with more than 20 carbohydrates has been isolated from human erythrocytes (61). The probable

precursor for this glycolipid is blood group H active megaloglycosphingolipid with 22 carbohydrates (62). Similar biosynthetic pathways for these two compounds are thought to exist (2).

Glycoproteins with blood group B activity are found in the urine, saliva, meconium and gastric juices of individuals with this blood type (33,34,35,62). Fucose containing oligosaccharides with B activity have been isolated from secretors with B blood type (69). The oligosaccharides are thought to be break down products from B active glycoproteins.

Similarities exist between the core oligosaccharides of B active glycoproteins and H active megaloglycosphingolipid which is thought to be the precursor for B active megaloglycosphingolipid (1,64). The oligosaccharide cores of both of these compounds contain lacto: Gal(β1-3)GlcNAc(β1-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc and lactoneo: Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc structures. These similarities may reflect common metabolic pathways for the oligosaccharide moieties of these molecules.

Blood Group A. Glycolipids and glycoproteins with terminal tetrasaccharide structures of GalNAc(α1-3)Gal[(1-2α)Fuc](β1-4)GlcNAc display blood group A activity. Four glycosphingolipid variants with A activity which have different carbohydrate compositions and core lengths have been isolated (65). Megaloglycosphingolipids with A activity and as many as 60 carbohydrate residues have been isolated from human erythrocytes (64,66). Blood group A^c glycolipid may have the same core oligosaccharide as H active megaloglycosphingolipid suggesting a common biosynthetic pathway (67). Unlike blood group B substances which have lacto and lactones structures, blood group A glycolipids only contain lactoneo sequences.

Another example of blood group specificity is illustrated by the Forssman heterophile antigen. This glycolipid antigen (globopentaglycosylceramide) is found in bacteria (68), as well as dog intestine (69), guinea pig tissues (70), and other mammalian tissue. In humans, very small amounts were found in gastrointestinal mucosa (71). Forssman antigen cross reacts extensively with blood group A antigens because this heterophile antigen has the same terminal GalNAc α1-3 moiety found in A active glycoconjugates (72,73).

Glycoproteins with blood group A activity have been isolated from human urine, saliva, meconium and gastric juices (33,34). These proteins share the terminal tetrasaccharide characteristic of blood group A glycolipids. Oligosaccharides, thought to be glycoprotein breakdown products, have also been isolated from the urine of secretors with this blood type (35).

III. Thy-1 Glycoconjugate

Introduction. The discovery of Thy-1 alloantigen in mice in 1963 opened up many areas of research addressing the interrelationship of various aspects of immunology and developmental biology. This review will seek to touch upon general characterization of Thy-1 and discuss in some detail the physical and immunochemical parameters which could possibly classify this antigen as a glycoconjugate.

During an investigation for a thymocyte immune cytolysis system, Reif and Allen (74) first discovered the presence of a strong non-histocompatibility (H-2) antigen on murine thymocytes. Using H-2 compatible strains of AKR and C3H mice, repeated injections of AKR thymocytes were given to C3H mice. The resulting isoantiserum was cytotoxic for AKR thymocytes and thymic derived leukemia cells when incubated with

a source of complement. The hallmark of this immunization procedure was the demonstration of allogeneic specificity i.e. C3H anti-AKR thymocyte serum was cytolytic for AKR thymocytes but not C3H thymocytes. Expanded studies by these authors (75) demonstrated that the reciprocal immunizations (C3H thymocytes injected into AKR) also displayed specificity for the immunogen (C3H thymocytes were lysed but not AKR). These antigens are now referred to as Thy-1.1 (formerly theta-AKR) and Thy-1.2 (formerly theta-C3H). Thy-1 antigens have since been found in all inbred mouse strains. AKR and a few related strains (AKR/J, AKR.B6, A.AL-old, BUB/BnJ, BDP/J, RFM/Un, PL/J, MA/MyJ and RF/J) carry the Thy-1^a allele (76) and express the Thy-1.1 antigen. C3H and the other inbred strains carry the Thy-1^b allele and express the Thy-1.2 antigen.

Itakura et al. (77) were able to determine that a genetic linkage existed, in mice, between the Thy-1 loci and d, a marker for dilute hair color found in linkage group II. Soon afterwards, this linkage was traced to chromosome 9 (78). Thy-1 is an autosomal gene with two codominant alleles. When a mating occurs between inbred parents with different Thy-1 types, the F_1 prodgeny are heterozygous and express both alleles. This codominant expression was demonstrated with C3H x AKR F_1 hybrids whose thymocytes were susceptible to lysis by C3H anti-AKR and AKR anti-C3H thymocyte serum (79). Geneticists have attempted to develop congenic strains for the Thy-1 locus. Acton et al. (80) feel that the AKR/J and AKR/Cum sublines are congenic. These strains, thought to differ only at the Thy-1 locus, will mount an anti-Thy-1 response against thymocytes and thymic leukemic cells of the other strain.

Thy-1 Determinants. The definition of any antigen depends upon the immunological assay used for its detection. Thy-1 has been

investigated in many assay systems producing varied results. It is, therefore, important to evaluate the following literature with these limitations in mind.

Most Thy-1 research has utilized some type of serology. Cytotoxicity, absorptions, antibody binding and immunofluorescent assays have been most widely used. The anti-Thy-1 serum used is very important because Thy-1 specificities change depending on the animal source. Thy-1 was originally defined as an alloantigen, antigenic variation found within a species such as histocompatibility antigens. These variations are recognized as foreign by members of the species who lack that allele. Thy-1 was originally defined as a murine alloantigen by murine antiserum (74). When murine cell surface Thy-1 is injected into another species, such as a rabbit, the allogeneic specificity defined by murine anti-Thy-1 serum is lost (81) and new Thy-1 determinants common to all mice are identified. Rabbit anti-mouse brain (anti-BA theta) serum recognizes two non-allogeneic Thy-1 determinants, a species specific determinant and a rat-mouse cross reacting determinant. This xenogenic serum fails to differentiate between Thy-1.1 and Thy-1.2 but recognizes other closely related Thy-1 determinants. The significance of the xenodeterminants and their relation to the alloantigen Thy-1 is unsettled at this date.

Distribution of Thy-1. Thy-1 is considered a marker antigen for identification of thymocytes and thymic derived lymphoyctes (81,82,83). Using the Thy-1 marker, Raff (83) showed that spleens and lymph nodes of mice treated with heterologous anti-lymphocyte serum (ALS) contained 80-90% fewer T cells than normal controls. Heterologous ALS has been shown to deplete peripheral or long lived T lymphocyte populations (83). Thymus tissue in both groups of mice was uneffected by ALS treatment.

Congenitally athymic nude mice have very small numbers of Thy-1 positive cells and are defiecint in most normal T-cell functions (84). Several other authors have demonstrated that T-mediated functions are associated with Thy-1 positive cells. Golub (81) found that anti-BA theta treatment abrogated the graft vs host reaction. In addition, Thy-1 and Ly positive cells were found to be responsible for antibody dependent cell-mediated cytotoxicity (85).

In adult mice, Thy-1 is present in large quantities on thymocytes (75,81,84,86) brain (75,81) and thymus derived leukemia cells (74,87). It is also present in lesser quantities on peripheral blood lymphocytes (84,88) lymph node and spleen cells (75,84) and non-lymphoid cells such as epidermal (89) and normal and neoplastic mammary cells (90,91). Thy-1 has not been found in bone marrow in adult mice (75,84).

Neonatal mice exhibit different Thy-1 distributions than do adult mice. Thymocytes are the only cell type which contains Thy-1 in adult levels (82). It is present in very small amounts in peripheral lymphoid tissues (84) and absent in the brain (75). These findings have prompted consideration of this antigen as one involved in differentiation.

The gray and white matter of adult murine brain expresses Thy-1 alloantigen (92) although its distribution is not uniform. The cerebral cortex (75,93) and synaptosomal fractions (93) have the highest concentrations in the brain. Support is given to Thy-1's surface membrane location by its absence from brain mitochondrial membranes (93).

Originally, Thy-1 alloantigen was thought to be restricted to mice. It is now apparent that a serologically indistinguishable antigen appears in the rat which is expressed only as the Thy-1.1 allele (94,95). Rat and mice show similar tissue distribuiton of this antigen with

slight variations. The highest concentrations are in thymocytes (95) and brain (94) with lesser amounts in the spleen (95) and on fibroblasts (96). Unlike mice, who display Thy-1 on peripheral lymphocytes but not bone marrow cells, rats express Thy-1 on bone marrow cells (97) but have negligable amounts on peripheral lymphocytes and in lymph nodes (95). Similar to mice however, neither neonatal mice and rats express Thy-1 at birth but reach adult levels in several weeks (94). The rat also has two non-allodeterminants associated with the Thy-1 molecule detectable with heterologous serum (98).

Heterologous serum has been used to detect Thy-1 associated xenodeterminants in other species. Goat anti-BA theta serum detected the highest levels of these determinants in rodent brain (mouse, rat, guinea pig) and smaller amounts in human, horse, cat and pig brain (99). Cross reactions between human thymus and brain with mouse lymphoid tissue have been reported by several investigators (100,101,102). Arndt et al. (100) report isolation and characterization of a molecule found in human thumus and brain which cross-reacts with the xenodeterminants of the mouse thymocyte brain antigen. Thy-1 alloantigen has not been identified in humans.

Differentiation and Thy-1 Antigen. The notable difference in the distribution of Thy-1 between neonate and adult led investigators to speculate about this marker antigens role in cell differentiation. Reif and Allen (82) suggested that Thy-1 was an example of postnatal immunological maturation because its appearance after birth paralleled the histological and physiological maturation of the brain.

Raff and Owen (84,103) while studying the development of thymus stem cells to peripheral T-derived lymphocytes, implicated Thy-1 as a

differentiation antigen. Thy-1 negative 14 day old CBA thymus cells were incubated in tissue culture. After four days, they became susceptible to cytolysis by anti-Thy-1.2 serum and complement as did normal 18 day old embryonic thymus cells. These authors also demonstrated that a second step in differentiation occured using an *in vivo* model. By grafting C3H (Thy-1.2) embryonic thymus cells into thymectomized bone marrow reconstituted AKR (Thy-1.1) mice, Raff and Owen were able to demonstrate the presence of Thy-1.2 and TL (another T associated differentiation antigen) positive cells in the spleens and lymph nodes of AKR mice 28 days later. They concluded that the maturation of peripheral T cells was both thymus derived and dependent.

Several investigators have used the Thy-1 marker quantitatively to differentiate sublines of T lymphocytes. Roelants et al. (104) used Thy-1 and surface immunoglobin (Ig) as a criteria to broadly classify all lymphocytes into five groups. One of these, Ig^- $Thy\text{-}1^+$ weak, was identified as an undifferentiated pre-thymic cell since it appeared in large numbers in nude and T-deprived mice and disappeared upon thymus graft transplant. Cantor et al. (88) and Olsson (105) agree that different amounts of cell surface Thy-1 identify functionally discrete subpopulations of peripheral T lymphocytes. More highly differentiated T lymphocyte (long lived memory cells) of the lymph nodes contained less Thy-1 (105) and more H-2 (84) than did short lived peripheral T lymphocytes of the spleen (105).

The use of thymic hormones have demonstrated the existance of a population of pre-thymic cells in bone marrow. Bone marrow cells separated using density gradients (106,107) and incubated with either thymosin (108) or thymopoietin (109) expressed cell surface Thy-1 as

detected by anti-Thy-1 serum and complement. Whether or not this is a reflection of true differentiation or simply a membrane rearrangement has yet to be decided.

Thy-1 Isolation and Biochemical Characterization.

The actual chemical composition of molecules which carry the Thy-1 alloantigenic determinant is presently unresolved. Evidence has been presented supporting a protein (110,111), glycoprotein (112,113,114,115, 116,117), or glycolipid (118,119,120,121) state for Thy-1 antigen. These conflicting results can be accounted for by the variety of isolation and characterization procedures used. Congenic and allogenic antisera as well as xenogenic antiserum have all been used in an attempt to isolate and identify the same antigen. Procedures using these different types of sera were applied to mouse brain, thymocytes, and lymphoma cells as well as rat brain and thymocytes. The methods used by each group should be kept in mind when interpreting the following results.

Protein Nature of Thy-1.

Atwell et al. (110) studied cell surface Thy-1 on CBA thymocytes. Tyrosine residues of cell surface proteins were labeled with the lactoperoxidase ^{125}I method. The membranes were solubilized in 10 M urea, 1.5 M acetic acid and dialyzed. Non-dialyzable protein was immunoprecipitated with homologous AKR anti-C3H thymocyte serum then goat anti-mouse serum. A 60,000 molecular weight band with Thy-1 activity was observed on disc gel electrophoresis in 5% sodium dodecyl sulfate (SDS) polyacrylamide. These authors did not attempt to determine if carbohydrate was present in the protein. This study (110) and a later one (122) characterized Thy-1 from thymocytes and found that Thy-1 was undetectable on B lymphocytes using anti-Thy-1 antiserum. Sauser et al. (123)

using a crude membrane source, claimed that Thy-1 mouse specific xenoantigen could be identified on SDS gels as a protein with a molecular weight of 40,000 daltons. Thy-1 alloantigen was postulated to be a glycolipid in close association with the xenoantigen on the protein. Papain and insolubilized protease treatment of Thy-1 destroyed this protein's inhibitory effects in a ^{51}Cr release cytotoxicity assay (111). The protein nature of Thy-1 is suggested by the above mentioned data.

Glycoprotein Nature of Thy-1.

Rat Thy-1. An antigen serologically indistinguishable from mouse Thy-1.1 alloantigen has been detected in rat brain and lymphoid cells (94,95). The Thy-1.2 allele, present in mice, has not been detected in rats (94). Rat thymocytes were estimated to have approximately the same quantity of cell surface Thy-1 as AKR (Thy-1.1) mouse thymocytes by using quantitative antibody binding studies (95).

Protein extractions require relatively mild isolation conditions to avoid denaturating these labile membrane components. In addition, a method to follow Thy-1 purification is necessary. The procedures described in this paragraph apply to all reports originating in the laboratory of A.F. Williams (112,114,115,124-128). Membrane Thy-1 was solubilized in a non-ionic detergent (Lubrol PX) or mild bile salts (deoxycholate) and assayed with an indirect radioactive binding assay (124). The amount of Thy-1 in a preparation was quantitated by the amount of inhibition it gave in the following assay. Heterologous rabbit anti-rat brain serum was mixed with glutaraldehyde fixed thymocytes and the extent of binding measured with ^{125}I labeled goat anti-rabbit Ig serum. It is important to note that this assay detects Thy-1 xenoantigens and not the Thy-1.1 alloantigen.

Rat thymocyte Thy-1 was solubilized in deoxycholate and isolated using gel filtration and antibody and lentil lectin (Lens culinaris) affinity chromatography (115). Two subclasses of thymocyte Thy-1 could be discriminated with the lentil lectin column. The lectin binding form of Thy-1 had a molecular weight of 25,000 daltons where as the unbound Thy-1 was closer to 27,000 daltons. Thy-1 was isolated from rat brain (125) which bound to lentil lectin and had a m.w. of 24,000 daltons using the same procedures mentioned above. Brain and thymocyte Thy-1 could not be distinguished antigenically using rabbit antiserum.

Characterization of rat brain and thymocyte Thy-1 molecules revealed the presence of three antigenic determinants. Rabbit anti-rat brain serum could detect two xenodeterminants, rat specific and rat-mouse cross reacting (126,127). The Thy-1.1 allodeterminant was also present and had the exact same tissue distributions as the xenoantigens. This observation and the fact that the three determinants were co-isolated, led these authors to suggest a close association, possibly on the same molecule, existed between these determinants.

Further studies into the chemical nature of the Thy-1 determinants led these investigators (112,114) to suggest that the protein portion of the molecule was responsible for its antigenicity. Brain and thymocyte Thy-1 were susceptible to heating and proteolysis. Thy-1 activity in the binding assay was destroyed by heating at 70-80^{o}C for 10 minutes. Pronase treatment at 37^{o}C for 24 hr destroyed Thy-1 activity although other proteolytic enzymes such as trypsin and papain did not. In addition, the amino acid compositions of brain and thymocyte Thy-1 were very similar even though heterogeniety was present in the carbohydrate compositions. Both sources of Thy-1 contained large amounts of mannose and

and small amounts of GlcNAc. GalNAc was found in brain but not thymocyte Thy-1. Differences also existed between these molecules in the amounts of Glc, Gal, Fuc, and NeuAc present. The different lentil lectin affinities of thymocyte Thy-1 might be explained by slight differences in their carbohydrate compositions. Differences in carbohydrate and similarities in protein components of the brain and thymocyte glycoproteins led these researchers to suggest the Thy-1 determinants were protein in nature (112,114).

The membrane nature of Thy-1 glycoprotein has created problems in determining its true molecular weight. This is not altogether unexpected as it has been noted that hydrophobic proteins bind large amounts of detergents (129). Up to 24% of the molecular weight of Thy-1 was estimated to be bound deoxycholate (130). Reevaluation of brain and thymocyte Thy-1 molecular weights results in molecular weights of 17,500 and 18,000 daltons respectively. When deoxycholate is removed with ethanol precipitation, Thy-1 forms on oligomer containing 16 glycoproteins monomers (130).

Mouse Thy-1. Arndt et al. (113) characterized a Thy-1 active protein isolated from mouse thymocytes and brain using urea-Nonidet P40 (NP-40) membrane solubilization and gel filtration. Three determinants (mouse specific, rat-mouse cross-reacting, Thy-1.1 allodeterminant) were present on this protein and were inseparable by isoelectic focusing. The observation that Thy-1 activity was destroyed by organic extraction but restored by auxillary lipids or non-ionic detergents led these authors to postulate that a protein lipid interaction was essential for Thy-1 antigenicity (113). Thy-1 activity was quantitated by an inhibition of cytotoxicity assay using rabbit anti-rat and mouse brain sera

as well as allogeneic anti-Thy-1.2 serum.

A 25,000 dalton glycoprotein from mouse brain has recently been characterized (128) utilizing procedures like those for rat brain glycoprotein (115). This glycoprotein has Thy-1 activity almost identical to rat brain Thy-1 glycoprotein. Two xenodeterminants (mouse specific, rat-mouse cross-reacting) were recognized in deoxycholate by heterologous rabbit serum. The Thy-1.2 allodeterminant was only detectable with allogeneic anti-Thy-1.2 serum in the absence of deoxycholate. These investigators address the affinity of allogeneic serum and the resulting problems it causes in absorption and binding assays. Antibodies specific for the Thy-1.2 allodeterminant are probably of low affinity and require multivalent determinants for binding. Only heterologous serum specific for Thy-1 xenodeterminants has a high enough affinity for use in binding and absorption studies (128).

Several murine lymphoma cell lines express cell surface Thy-1 antigen (75). Johnson et al. (131) subjected S49.1 lymphoma target cells to neuraminidase and trypsin treatments in an effort to characterize membrane bound Thy-1. Target cells treated with either enzyme were no longer susceptible to lysis by anti-Thy-1.2 serum, leading these authors to suggest that sialic acid may be part of the Thy-1.2 determinant on a glycoprotein molecule. Lymphoma Thy-1.1 glycoprotein has been isolated from kilogram quantities of BW5147 lymphoma cells (116) using procedures previously discussed for rat Thy-1 glycoprotein (115). This molecule has a molecular weight of 25,000 daltons and expresses the three characteristic Thy-1 glycoprotein determinants.

Naturally existing Thy-1 negative mutants have been observed in lymphoma cell populations by Hyman and Stallings (132) using indirect

selection techniques. Cell hybridizations were preformed with different Thy-1 negative mutants and these cell lines classified by their ability to complement each other for the expression of Thy-1. The genetic defects in these complementation classes were suspected to be defective structural or regulator genes for Thy-1. Thy-1 negative cells were later shown (133) to have incompletely glycosylated Thy-1 glycoprotein (T-25) while other cell glycoproteins were normal. This prompted Trowbridge and Hyman to suggest that Thy-1 genes code for the glycosyltransferases necessary for assembly of the T-25 oligosaccharide moieties and that Thy-1 antigenicity is carbohydrate determined.

T-25 glycoprotein was ^{125}I lactoperoxidase labeled and isolated by deoxycholate solubilization, pea lectin affinity chromatography and gel filtration (117,134). Immunoprecipitation was then preformed with rabbit anti-mouse thymocyte or anti-mouse T lymphoma sera or anti-rat brain Thy-1 glycoprotein serum. All three sera reacted with the same 25,000 dalton glycoprotein. T-25 was also shown to be free of glycolipid contamination because it failed to incorporate [^{3}H]palmitate (117). Carbohydrate moieties on glycoproteins appear to play a role in the membrane transport and half life of these molecules (135). Lymphoma cells with Thy-1 negative phenotypes produce an incomplete T-25 molecule with small amounts of carbohydrate which are rapidly degraded in the cell cytoplasm. Immunoelectron microscopy showed that incomplete T-25 molecules were not present on the cell surface but accumulated in the cytoplasm (135).

Structural analysis of Thy-1 glycoproteins have just begun. The amino acid compositions, but not sequences, have been determined for rat Thy-1 glycoprotein (114). These studies have shown that membrane

Thy-1 is a small asymmetrical hydrophobic glycoprotein. Carbohydrate labeling indicates that the oligosaccharide moieties contain a high percentage of mannose. This has been substantiated with murine lymphoma Thy-1 which also contains a large percentage of mannose (136). Glycoprotein classifications have been discussed previously in the glycoconjugate section. It was noted that the high mannose types of oligosaccharide moieties are found on most plasma membrane glycoproteins. This type of glycoprotein has oligosaccharide chains that originate with a glycopeptide bond between GlcNAc and the amide linkage of asparagine. Barclay et al. (114) found substantial quantities of GlcNAc in rat Thy-1. This linkage is referred to as an N-glycosidic linkage. It can be differentiated from O-glycosidic linkages found in serum and mucus glycoproteins by its resistance to mild alkali (0.1N NaOH at 37^{o} for 72 hr). T-25 glycopeptide bonds were found to be resistant to these conditions by Trowbridge et al. (136) supporting the presence of N-glycosidic linkages between GlcNAc and asparagine.

Glycolipid Nature of Thy-1.

The first report on the chemical nature of Thy-1 (79) described this antigen as non-dialyzable, unaffected by deoxycholate but sensitive to lipid solvents. Reif and Allen suggested that Thy-1 was a lipoprotein. Vitetta et al. (119) succeeded in isolating a Thy-1 membrane complex from mouse thymocytes by surface protein radioiodination, freeze thaw lysis, and immunoprecipitation with congenic anti-Thy-1 serum. After density gradient centrifugation, the Thy-1 complex had a lower density than protein since it was found in the lipoprotein area. Non-ionic detergent (NP-40) abolished the antigenicity of the Thy-1 complex. Thymocytes were incubated with several radioactive

precursors, but only [^{3}H]galactose ended up in the Thy-1 active complex. Labeled amino acids were not incorporated into the Thy-1 complex. Polyacrylamide gel electrophoresis of Thy-1 labeled with [^{3}H]galactose resulted in diffuse radioactivity between the major 35,000 dalton peak and and the tracking dye (glycolipid area). From these results, Vitetta et al. (119) suggested that a protein glycolipid complex was responsible for Thy-1 activity.

A glycolipid nature for Thy-1 was supported by the work of two budding young scientists named Esselman and Miller (120). Mouse thymocytes and brain tissues were extracted with chloroform-methanol mixtures and the whole lipid extract was subjected to a biphasic (Folch) partition. The thymocytotoxicity of rabbit anti-mouse brain serum (BA theta) could be inhibited when absorbed with material from the ganglioside rich Folch upper phase. Further separation of the upper phase with thin layer chromatography (TLC) revealed that material corresponding to G_{D1b} ganglioside, when reconstituted in cholestrol and lecithin, could inhibit anti-BA theta serum. Esselman and Miller (120) claimed that G_{D1b} ganglioside was BA theta antigen (a Thy-1 xenodeterminant). Using a different ganglioside isolation procedure than Esselman and Miller, Arndt et al. (113) were unable to reduce the cytotoxicity of anti-BA theta serum with G_{D1b} ganglioside. However, organic extraction of a protein with Thy-1 activity resulted in a major loss of antigenicity. Because the activity could be restored with auxillary lipids (cholestrol:lecithin) or NP-40 these investigators suggested that a protein lipid interaction was necessary for Thy-1 antigenicity.

Miller and Esselman (137) later reported that G_{M1} ganglioside, isolated by TLC, in liposome form (cholestrol:lecithin) was able to

inhibit the thymocytotoxicity of allogeneic anti-Thy-1 sera. These authors showed that G_{M1} liposomes, presumably by interacting with B cells, also depressed the SRBC responses of spleen cell cultures. This effect was shown to be Thy-1 allotype specific. Only G_{M1} from thymocytes or brain of the same Thy-1 type as the spleen cells could "modulate" the spleen cell SRBC responses. This effect could be specifically inhibited by preincubation of G_{M1} liposomes with alloantisera before addition to the cultures.

Other investigators disputed the finding that G_{M1} and Thy-1 were one and the same. Using indirect immunofluorescence, Stein-Douglas et al. (138) observed that antibody to purified G_{M1} reacted with thymocytes and peripheral lymphocytes independent of Thy-1 type. The allogeneic specificity characteristic of Thy-1 was not observed in these experiments indicating Thy-1 and G_{M1} were separate entities. G_{M1} ganglioside is the primary receptor for cholera toxin (CT) and choleragen, the binding subunit of CT (139). Cocapping and blocking experiments with CT and anti-Thy-1 allosera have failed to clarify the relationship between G_{M1} and Thy-1. Milewicz et al. (107) preincubated C3H thymocytes with CT. The observation that these cells displayed a dramatic decrease in susceptibility to anti-Thy-1.2 serum prompted these authors to suggest that close association exists between G_{M1} and Thy-1. The blocking effect could be a result of G_{M1} and Thy-1 being the same or two very closely associated molecules. Thiele et al. (140) using immunofluorescent capping procedures, observed a common ligand induced redistribution of Thy-1 and G_{M1}. Choleragen did not block the binding of anti-Thy-1.2 serum, however, suggesting to them that while closely associated, Thy-1 and G_{M1} were spatially separated. In contrast, DeCicco and Graves (141)

observed independent capping of fluorescent labeled anti-Thy-1 serum and CT. A possible explanation of these conflicting results is offered by Bourguignon et al. (142). Unrelated thymocyte membrane molecules (H-2, TL, Thy-1) co-migrated with T-200 glycoprotein when capping was induced with anti-T-200 antibody. These authors suggested that H-2 may passively "tow" other molecules along during the capping phenomena. This effect could vary depending on the assay conditions and thus be responsible for the conflicting capping results.

Recently, Wang et al. (121) demonstrated that brain and thymocyte Thy-1 activity may be separated from G_{M1} with TLC using an alternate solvent system. The Thy-1 activity appeared to be present as a minor G_{M1} contaminant. The authors suggest a glycolipid nature for Thy-1 with properties similar to gangliosides. An immune response assay, originally developed by Fuji and Milgrom (143) was modified for *in vitro* culture with isolated Thy-1 antigens. Most interesting was the ability of the anti-Thy-1 PFC assay (121) to detect both glycolipid and glycoprotein Thy-1 antigens. Wang et al. suggest that these observations strongly support the contention that Thy-1 specificity is determined by the carbohydrate moiety which is shared by glycolipid and glycoprotein carriers.

Thy-1 Glycoconjugate.

The literature reviewed in this section indicates that the chemical nature of Thy-1 antigen is still a matter of controversy. Evidence supporting glycolipid, glycoprotein and other compositions have been discussed. Methodology and various tissue sources might explain some of the different results but mounting evidence seems to support the coexistance of glycolipid and glycoprotein which share Thy-1 allotypic properties. The anti-Thy-1 PFC assay can detect Thy-1 antigenicity in both

glycolipid and glycoprotein structures. A common antigenic moiety such as carbohydrate may determine Thy-1 specificity. Blood group specificities are determined by terminal carbohydrate moieties shared by glycolipids and glycoproteins (49,50). Tonegawa and Hakomori (144) found glycoproteins with terminal carbohydrate chains identical to the major globosides and gangliosides. These "globo- and ganglioproteins" cross react extensively with anti-globoside and anti-ganglioside sera. A common carbohydrate biosynthetic pathway may link the ganglioproteins and gangliosides as well. Viral induced transformation of cells resulted in the same carbohydrate deletions in both the glycoproteins and glycolipids isolated from these cells (144). Multienzyme glycosyltransferase complexes responsible for the synthesis of a particular oligosaccharide chain were proposed by Roseman (13). As discussed earlier, glycosyltransferase complexes may be shared by glycolipids and glycoproteins. The Thy-1 genes might code for such a complex capable of synthesizing an oligosaccharide on both glycolipid and glycoprotein.

Immune Response to Thy-1.

All inbred strains of mice express either Thy-1.1 or Thy-1.2 alloantigen on their lymphoid and brain tissue. A pair of codominant alleles on chromosome 9 control the surface expression of this antigen (77,78). Inbred mice have the ability to mount a humoral immune response when challenged with the Thy-1 allele they lack (74,76). The magnitude of the anti-Thy-1 response was proposed to be genetically determined by Fuji et al. (145) following their observation that different inbred mouse strains challenged with the same dose of thymocyte antigen had widely varying anti-Thy-1 titers. The immune response to Thy-1 is controlled by a pair of codominant alleles in close association with the H-2 locus

(145). Investigation of this antibody response has established serological and immune response assays for Thy-1 antigen.

Serological Assays for Thy-1. The majority of assays for Thy-1 utilize antiserum in some way. Assays described in the previous section include thymocytotoxicity (74,75,79,84), immunofluorescence (140,141), antibody binding (115), and inhibition of these assays in which the anti-serum is absorbed with tissue or mixes with isolated antigens.

Anti-Thy-1 serum is produced in animals capable of an anti-Thy-1 immune response after challenge with Thy-1 containing tissues (usually thymocytes or brain) or purified antigens. Congenic or allogeneic antisera is made after an intravenous injection of mouse thymocytes of one Thy-1 type into a mouse bearing the other Thy-1 type (74,80). The resulting sera recognizes one of the Thy-1 (1.1 or 1.2) allodeterminants (79). Heterologous antiserum is produced in another species, such as goat or rabbit, after an injection of Thy-1 containing mouse or rat tissue (99). These sera cannot recognize strain specific allodeterminants, but rather species specific determinants such as the Thy-1 xenodeterminants (rat or mouse specific and rat-mouse cross-reacting). The assays utilizing these sera are limited by the specificity and affinity of the antibodies within them. For example, it is important to note that congenic or allogeneic sera have a low affinity for Thy-1 alloantigen which makes these sera suitable for cytolysis assays but limits their application in procedures such as antibody binding and absorption (128, 146).

Immune Response Assay for Thy-1. Cells producing antibody to Thy-1 can be used directly to detect Thy-1 alloantigen. Fuji et al. (147) discovered that after an injection of a mouse of one Thy-1 type with mouse

thymocytes of another Thy-1 type, the challenged spleen cells from the immunized mouse could be removed, mixed with agar and target thymocytes, of the immunizing Thy-1 type, on a microscope slide, and plaques could be detected where antibody forming cells had lysed the target cells in the presence of rabbit complement. Plaques which were clear areas against a cloudy white background, were visualized after fixing the slides in ethanol and drying them. This procedure is a modification of the plaque forming cell (PFC) assay devised by Jerne and Nordin (148) to detect antibody responses to sheep red blood cells (SRBC). The anti-Thy-1 PFC assay detects alloantigens on nucleated cells. Good correlations were demonstrated between the magnitude and kinetics of the anti-Thy-1 PFC response and anti-Thy-1 serum titers (145).

Specificity of the Anti-Thy-1 PFC Assay. Several methods have been used to show that this assay detects Thy-1 alloantigen. PFC are only observed when immunizing and target cells of the same Thy-1 type are used to challenge and assay, respectively, spleen cells of the other Thy-1 type (145,149). Significant PFC responses were never detected when the thymocytes used for immunization and the responding spleen cells lacked identity at the H-2 loci but shared Thy-1 allotype (149,150,151, 152). Only when lymphoblastoid cells, which have higher concentrations of surface H-2 than thymocytes, were used as target cells could plaques directed to H-2 antigens be detected (149). The observation that only Thy-1 bearing immunizing agents elicited an anti-Thy-1 PFC response and the best target cells were those with the highest concetration of surface Thy-1 (145,149) further support the recognition of Thy-1 antigen by the PFC assay.

Magnitude and Kinetics of the Primary and Secondary Responses. Thy-1.2 mice which mount a primary anti-Thy-1.1 response were classified by Fuji et al. (145) according to the magnitude of their PFC responses measured in the anti-Thy-1.1 PFC assay. High responders were mice with greater than 10^4 PFC/spleen, low responders has less than 10^3 PFC/spleen, and all mice between these values were intermediate responders. The primary responses of Thy-1.1 mice to the Thy-1.2 allele were classified as either high (> 10^3 PFC/spleen) or low (< 10^3 PFC/spleen) responders in a similar manner (152). A primary response can be detected two days following immunization and reaches a maximum in four to seven days for both high and low responders. The primary response can still be detected for three weeks in high responders but lasts for only 10 days in low responders (150). High and low responder secondary responses were not greatly different because low responders had a larger secondary than primary response and most high responders had a smaller secondary than primary response (145). IgM antibody was measured in this assay as indicated by its sensitivity to 2-mercaptoethanol (150).

Genetic Control of the Anti-Thy-1 PFC Response. Immune responsiveness is thought to be a reflection of the number of relevant precursor cells an animal has. The number of precursor cells an animal has is genetically determined (153,154). Genetic control of the anti-Thy-1 response was indicated by a wide variation of serum titers and anti-Thy-1 PFC responses from different strains of mice (76,145,155). Zaleski and Klein (155) used special hybrid mouse strains to show that two codominant alleles (IR Thy-1A and IR Thy-1B), closely linked to the H-2 locus, are responsible for the magnitude of the anti-Thy-1 response. These authors suggested that minor effects were also exerted by the IR-5 locus which

is outside of the H-2 region. The picture is further complicated by the observation that unrelated alloantigens play a role in the magnitude of the anti-Thy-1 response (76,156).

In Vitro Modification of the Anti-Thy-1 PFC Assay. An *in vitro* modification of the original *in vivo* anti-Thy-1 PFC assay was developed for the study of naturally occuring autoantibodies to thymocytes (143). Normal spleen cells were stimulated in tissue culture with cell free thymocyte supernatants and six days later plaques were enumerated as previously described (147). Small quantities of anti-thymocyte antibody were produced by the spleen cells against syngeneic and all other thymocyte target cells tested (143).

Lake (157) used a similar *in vitro* assay to look at shed Thy-1 complexes in thymocyte supernatants. The *in vitro* culture methods of Mishell and Dutton (158) were combined with the plaque assay developed by Fuji et al. (147) to produce an *in vitro* system with the same Thy-1 specificity described previously for the *in vivo* procedure. This assay measured a peak response at four to five days which would disappear three days later (157). A secondary response could be detected if spleens from mice previously primed once with thymocytes were challenged *in vitro* with thymocyte culture media. No secondary response was observed when these same cells were challenged with whole thymocytes (157).

This assay was utilized by Freimuth et al. (159) to characterize the shedding phenomena in Thy-1 bearing lymphoblastoid cells. The PFC response was directed to Thy-1 present in high molecular weight membrane complexes release from the cells *in vitro*. Shedding kinetics were also followed with the PFC assay.

Wang et al. (121) found the *in vitro* anti-Thy-1 PFC assay useful for

measuring PFC to isolated Thy-1 active glycolipid and glycoprotein antigens. The magnitude of the response to these antigens was smaller, though statistically significant, than that for thymocyte shed media. This might be expected because lower molecular weight substances are not as immunogenic as those with higher molecular weights.

Biological Significance of Thy-1.

Thy-1 alloantigen is expressed during the differentiation of thymus derived lymphocytes and serves as a marker for these cells. Its presence can be induced in bone marrow populations through the action of thymic hormones. Several authors proposed that Thy-1 plays a role in the regulation of T-cell differentiation or in an immune recognition process in T-cells (84,89,103,118).

Thy-1 is also associated with the shedding process in cultured lymphoid and non-lymphoid cells. It has been found in large membrane complexes from lymphoblastoid cell supernatants (158) as well as the supernatants of thymocytes (118). Vitetta (118) suggested Thy-1 exists in the membrane periphery rather than as an integral part since it is shed independently of H-2. Mammary cells infected with mammary tumor virus (MTv) release viral particles whose envelopes contain both H-2 and Thy-1 (91). Non-MTv infected cells do not release either Thy-1 or H-2. Whether or not Thy-1 plays a significant role in this process depends on the importance of shedding itself. The shedding phenomena must be better understood before it can be decided if this is simply an *in vitro* artifact or whether it represents an important function such as cellular communication.

Esselman and Miller (137,160,161) found that Thy-1 active glycolipids could modulate the immune response of spleen cell cultures to SRBC antigens. Addition of Thy-1 active glycolipids, in liposome form, to spleen

cell cultures resulted in a drastic decrease of anti-SRBC PFC responses. This effect, which was directed toward B target cells, was a temporary one lasting only a few days and was shown to be Thy-1 specific. Esselman and Miller postulated that a Thy-1 liposomal interaction with B cells might be a mechanism to protect these cells from tolerance due to antigen overload (137,160). Although no specific role for this antigen has been agreed upon, it will continue to serve as an important research tool. Its usefulness as a marker for T-cells is already apparent and its application to such phenomena as differentiation, shedding and immune modulation will continue to shed light on these topics. Examination of the glycoconjugate nature of Thy-1 may also serve to further the understanding of the relationship between glycolipid and glycoprotein biosynthesis.

INTRODUCTION TO THE EXPERIMENTAL REPORT

The Thy-1 alloantigen is the product of codominant alleles on chromosome 9 in mice (77,78). All inbred mouse strains are homozygous at this locus and express either the Thy-1.1 (AKR and a few related strains or Thy-1.2 (C3H and most other strains) allele. Many attempts have been made to characterize the molecule which carries Thy-1 antigenic activity. We previously proposed that the alloantigenic determinants in brain and thymic lymphocytes were carried by glycolipids as well as glycoproteins (121). The Thy-1 glycolipid had properties similar to gangliosides and was isolated and partially purified with column chromatography and thin layer chromatography (TLC). Thy-1 antigenicity was assayed with a modified *in vitro* PFC technique originally developed for detecting alloantigenic differences on nucleated cells by Fuji and Milgrom (143). This assay demonstrated the allogenic specificity of both glycoprotein and glycolipid forms of Thy-1.

Thy-1 antigenicity has been claimed to be present on a protein (110,111) or glycoprotein molecule (113,115,117). The actual composition of the Thy-1 alloantigenic determinant has yet to be established for the glycoprotein. Barclay et al. (114) suggest the rat Thy-1 specificity is of a protein nature because of similarities in amino acid composition between brain and thymocyte Thy-1, as well as the heat lability and proteolytic susceptibility of the molecule. Arndt et al. (115) established that a protein lipid interaction is necessary for Thy-1 antigenic activity. Trowbridge and Hyman (117) have used Thy-1 negative mutant lymphoma cell lines to suggest that carbohydrates of T25 (Thy-1 glycoprotein) are responsible for its antigenicity.

We now report the radiolabeling of brain and lymphoma Thy-1 active glycolipids with a sialic acid precursor (N-acetylmannosamine) and a sphingosine precursor (palmitic acid), as well as the isolation of Thy-1 glycolipids by two dimensional TLC. The ganglioside nature of Thy-1 glycolipid is strongly suggested by interactions with DEAE cellulose ion-exchange chromatography, and neuraminidase treatment.

MATERIALS AND METHODS

Radiolabeling of Brain Glycolipids. Thy-1 glycolipid was labeled biosynthetically using a previously described method (19). One litter of 7 day old pups (5-7 mice) of either AKR/J (H-2^k, Thy-1.1) or ICR Swiss (Thy-1.2) mice were used for each preparation. AKR mice were obtained from Jackson Laboratories, Bar Harbor, ME and ICR Swiss mice were obtained from Spartan Research Animals, Inc., Haslett, MI. Each mouse pup was injected intracranially with 8 ul of sterile saline solution containing 5 uCi [1-^{14}C]N-acetylmannosamine, 54.5 mCi/mmol (New England Nuclear, Boston, MA). This solution was injected into both sides of the head at a point 1-2 mm anterior to the intrauricular line and 2-3 mm lateral to the midline with a 10 ul Hamilton syringe (Hamilton Company Inc., Whitting, CA). The tip of the needle was introduced only far enough to completely submerge the bevel of the tip below the surface of the skull. The pups were then returned to their mothers. Two days later the brains were removed and pooled for each group and the glycolipids isolated as described below. Incorporation of ManNAc exclusively into the sialic acid of gangliosides was confirmed by neuraminidase hydrolysis.

Radiolabeling of Lymphoma Cells. BW5147 (AKR/J, Thy-1.1, H-2^k) and S.49.1 (BALB/c, Thy-1.2, H-2^d) murine lymphoma cell lines were

obtained from the Salk Institute Cell Distribution Center (LaJolla, CA). Cells to be labeled for glycolipids in culture were washed once, then incubated in fresh Dulbecco's modified Eagles medium (D-MEM, Grand Island Biological Co., Grand Island, NY) containing 1000 mg/l glucose, with 10% heat inactivated fetal calf serum (FCS, Grand Island Biological Co.) and and either 2.0 uCi/ml [1-^{14}C]N-acetylmannosamine, 54.5 mCi/mmol, or 2.0 uCi/ml [1-^{14}C]palmitic acid, 30 mCi/mmol (International Chemical and Nuclear Corp., Irvine, CA). Cells were cultured at 37^{o}C in flasks at a concentration of 5 x 10^5 cells/ml. The cells reached a concentration of 2 x 10^6 cells/ml after 48 hours. If viability as determined by trypan blue exclusion was greater than 95%, the cells were washed once with phosphate buffered saline (PBS) and the pellet was extracted as described below.

Glycolipid Preparations. Biosynthetically radiolabeled glycolipids were isolated from brain and lymphoma cells with chloroform-methanol (C:M) mixtures (162). The total lipid extract was subjected to a Folch partition (163) and the ganglioside rich upper phase was dried *in vacuo* and hydrolysed with mild alkali using 0.3 N NaOH in methanol-chloroform (1:1, v/v) for one-half hour at room temperature. This mixture was neutralized with glacial acetic acid, evaporated, resuspended in distilled H_2O and dialyzed for 48 hr at 4^{o} against several changes of distilled water. The dialyzed samples were checked at this point for radioactive incorporation by liquid scintillation spectrometry. Upper phase samples from brain were applied to TLC plates and column chromatography for further purification. Lymphoma upper phase samples were further purified on TLC plates.

Column Purification of Thy-1 Glycolipid. Unlabeled brain glycolipids

were isolated as described above. Dialyzed upper phase brain glycolipids from AKR/J or C3H/HeJ (Jackson Labs, Bar Harbor, ME) mice were separated by column chromatography using Anasil S (Analabs Inc., North Haven, CT) with chloroform-methanol-water (C:M:W) mixtures (6). Column fractions were pooled and prepared for Thy-1 testing, using the anti-Thy-1 PFC assay as described below.

Thin Layer Chromatography. Both one and two dimensional preparative TLC were used to purify Thy-1 glycolipid. All experiments were performed at 4°C using Silica Gel 60 TLC plates (E. Merck, Darnstadt, West Germany). Dialyzed upper phase samples of brain and lymphoma cells with between 30-50,000 cpm were used for each TLC plate. All radiolabeled samples were chromatographed in parallel with mouse brain ganglioside standards extracted as described above. These standards were identified by TLC R_f migrations compared to compounds previously analyzed using quantitative gas-liquid chromatography. The TLC developed standards were visualized with I_2 vapors and served as a reference to compare with the autoradiographic exposures of the brain and lymphoma samples. Isolation of Thy-1 glycolipid from radiolabeled ICR brain involved three sequential one dimensional TLC steps. The labeled material was spotted on a TLC plate and first run in solvent 1 (C:M:W, 50:40:9, containing 0.02% [w/v] $CaCl_2 \cdot 2H_2O$). The radiolabeled samples were eluted from areas corresponding to ganglioside standard G_{D1a} and slightly above it (but not including G_{D3}) as described by Esselman et al. (162). This eluted material was spotted on a second TLC plate and developed in solvent 2 (C:M:W:NH_4OH, 60:35:6.6:1.4, v/v/v/v). From the second plate a band detected by autoradiography and corresponding to standard G_{D1a} was isolated as well as the band directly above G_{D1a}. These two samples were run in parallel

on a third TLC plate with mouse brain ganglioside standards in solvent 1.

A two dimensional TLC system was developed to attempt the purification of Thy-1 glycolipid using only one plate. The radiolabeled glycolipid material (brain or lymphoma) was spotted in a small area in the corner of the plate. Mouse brain ganglioside standards were run in each dimension in troughs separated from the test sample. The plate was first developed twice in one dimension in solvent 2. Then it was developed once in the other dimension in solvent 1. Each plate was air dried for 1 hr then dried *in vacuo* for 45 min (between runs) to ensure dryness.

Autoradiography. Each plate was covered with a 3 x 10 in. sheet of Kodak SB-5 X-ray film (Eastman Kodak Co., Rochester, NY) and kept at $4^{o}C$ in the dark until developed. The time of exposure varied between 12 days and 3 months depending upon the specific activity of the compounds involved. The films were developed in Kodak X-ray Developer-Replenished (#146-5327) and fixed in Kodak Rapid Fix (#146-4106). The developed films were placed on their respective plates and the radiolabeled glycolipids were selected for testing by comparing the relative position of the spots on the films to the ganglioside standards visualized with I_2 vapors on the side of the TLC plates. Selected areas on the TLC plates directly under the spots on the films were eluted from the silica gel as described above and tested in the anti-Thy-1 PFC assay.

Thy-1 Chemical and Enzymatic Treatments. Clostridium perfringens neuraminidase (Sigma, St. Louis, MO) 0.1 units, was added to dried glycolipid in acetate buffer pH 4.5 and incubated for one hour at $37^{o}C$. The sample was heated to $100^{o}C$ for 15 minutes, dried, extracted with C:M (2:1) and tested as described below. DEAE cellulose chromatography was performed by application of a small volume of glycolipid in C:M (1:1) to a

3 ml column of DEAE cellulose, acetate form, followed by elution with 5 column volumes of C:M:W (60:40:8). Bound glycolipids were eluted with 5 column volumes of chloroform-methanol-ammonium acetate (162). The samples were dried and tested in the PFC assay as described below. Mild HCl treatment was performed as previously described with 0.1 N HCl at 80°C for 30 minutes (162). After hydrolysis the samples were neutralized with 0.1 N NaOH dried and tested in the PFC assay.

Anti-Thy-1 PFC Assay. Fuji and Milgrom (143) originally developed an *in vitro* PFC assay which detected Thy-1 alloantigen on whole thymocytes. A modified version, used here, has previously been described in detail and found effective for measuring a secondary immune response to isolated glycolipid and glycoprotein Thy-1 alloantigens (121).

The spleen cells for the anti-Thy-1.1 PFC assay were obtained from C3H/HeJ mice primed with a single intravenous injection of 4×10^7 AKR/J thymus cells in Eagles minimal essential medium (MEM) 2-3 weeks before use. Primed AKR/J spleen cells for the anti-Thy-1.2 PFC assay were obtained from mice injected once with 4×10^7 C3H/HeJ thymuc cells in MEM. One ml containing 2×10^7 viable spleen cells of either strain in medium CMRL 1066 supplemented with 15% FCS (both from Grand Island Biological Co.) was placed in the inner chamber of a Marbrook culture vessel. Glycolipid antigens to be tested were dried of organic solvents in sterile vials, resuspended, and incubated in 0.5 ml CMRL 1066 containing 15% FCS for one hour at 37°C before addition to either AKR/J or C3H/HeJ spleen cells. The spleen cells were incubated with antigens for 4 days at 37°C in a humid 8% CO_2 atmosphere. Cells were removed from the inner chamber and the concentration of viable cells in each experimental group determined. Target cells for the anti-Thy-1.1 PFC assay were

obtained from the thymuses of 10-16 week old AKR/J mice. Likewise, C3H/HeJ thymus target cells were used in the anti-Thy-1.2 PFC assay. Cultured C3H/HeJ spleen cell were centrifuged and the pellets were resuspended in 0.1 ml MEM containing 2×10^7 viable AKR/J thymocytes. AKR/J spleen cell pellets were similarly resuspended in 0.1 ml MEM containing C3H/HeJ thymocytes. Tubes containing 0.3 ml of 0.6% agarose (Induboise Industrie Biologique, Francais) dissolved in MEM with 0.5 mg DEAE-dextran/ml (Pharmacia Fine Chemicals, Piscataway, NY) were maintained in a 50-53°C water bath. Each spleen-thymocyte cell suspension (20°C) was added to the warmed agarose solution, vortexed, and immediately poured on a microscope slide previously dipped in a 0.1% agarose solution. After gelation, the slides were incubated upside down in plastic slide trays and D-MEM/FCS was added to cover each slide. The slides were incubated for 5-5.5 hr at 37°C in a humid 8% CO_2 atmosphere. Rabbit complement (Grand Island Biological Co.), in D-MEM/FCS (1:10 v/v) was added and the slides were further incubated for 45 min.

Plaques were determined by a staining technique (164) in which slides were drained and 0.2% trypan blue in 0.15 M PBS (pH 7.2) was added for 20 min. at 20°C. The slides were rinsed twice in PBS and the dark trypan blue stained plaques were counted using a dissecting microscope. The PFC response for each glycolipid antigen was an average of five cultures. Standard errors were calculated and the Student's t test applied to determine statistical significance. All p values were less than 0.05. A positive Thy-1 control and a negative medium control were included in each assay. The background PFC response of the medium control was subtracted from glycolipid PFC responses.

RESULTS

Mouse brain glycolipids were separated by column chromatography with Anasil S using previously published procedures as described in the Materials and Methods. Glycolipid fractions were pooled and tested for Thy-1 antigenicity using the anti-Thy-1 PFC assay. Thy-1 glycolipid was previously shown to be present only in pooled fractions containing G_{M1} ganglioside (121).

Thy-1.1 and 1.2 glycolipid were characterized by one dimensional TLC. Unlabeled column G_{M1} fractions from AKR/J (Thy-1.1) and C3H/HeJ (Thy-1.2) brains were mixed and applied to a thin layer plate next to a mouse brain ganglioside standard. After development in solvent 1, fractions corresponding to the brain ganglioside standards (illustrated in Figure 1) were eluted from the silica gel, divided, and assayed for Thy-1.1 and 1.2 antigenicity using the anti-Thy-1 PFC assays (Figure 1). Individual Thy-1 allotypes could be assayed in mixtures because this assay can identify either the Thy-1.1 or Thy-1.2 allotype specifically (sse below). AKR spleen cells used in the anti-Thy-1.2 PFC assay respond only to antigens of the Thy-1.2 allotype and not to those with the Thy-1.1 allotype. C3H spleen cells used in the anti-Thy-1.1 PFC assay, likewise, only respond to the Thy-1.1 allotype (121). Thy-1 glycolipid was not associated with G_{M1} ganglioside after development in solvent 1. Thy-1.1 glycolipid was found only in fraction 4, Thy-1.2 glycolipid was present only in fraction 3, and G_{M1} in fraction 9. Thus, in solvent 1, brain Thy-1.1 glycolipid migrates slightly faster than Thy-1.2 glycolipid and ganglioside standard G_{D1a}.

Thy-1 glycolipid could not be visualized on the TLC plate in Figure 1 using I_2 vapors or spray resgents because of the minute quantities

Figure 1. Thin Layer Chromatography of Thy-1. Column G_{M1} fractions containing Thy-1 glycolipid from AKR and C3H brain were mixed and chromatographed in solvent 1. The sample was eluted in fractions, divided, and assayed in the anti-Thy-1.1 and anti-Thy-1.2 PFC assays. Standard mouse brain gangliosides were run in a parallel channel and is shown below the graph. The chromatography proceeded from left to right (arrow).

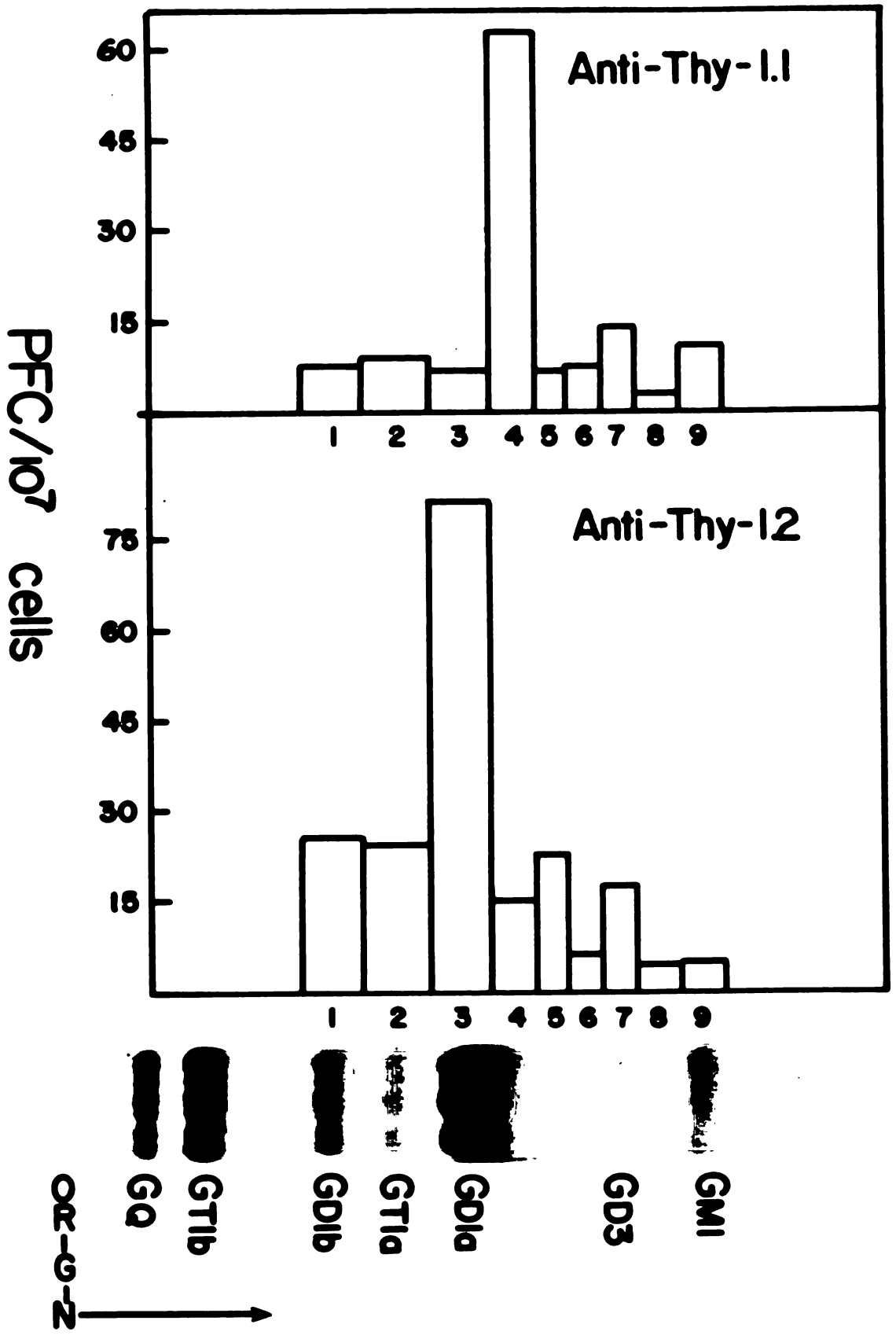
Anti-Thy-1.1
Anti-Thy-12
PFC/10⁷ cells
60
45
30
15
75
60
45
30
15
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
OR-G-Z
GQ
GTIb
GDIb
GTIa
GDIa
GD3
GMI

Figure 1.

Figure 2. One Dimensional TLC of Radiolabeled Thy-1 Glycolipid Using Sequential Solvents. ICR brain ganglioside, radiolabeled with[1-^{14}C] ManNAc, were chromatographed in solvent 1 (step). The Thy-1 active area (A) was eluted and chromatographed in solvent 2 (step 2). The Thy-1 active area (B) was eluted and chromatographed again in solvent 1 (step 3). The Thy-1.2 active band is shown by the arrow in step 3. Standard gangliosides are shown in step 3. All gangliosides in this figure were visualized with autoradiography.

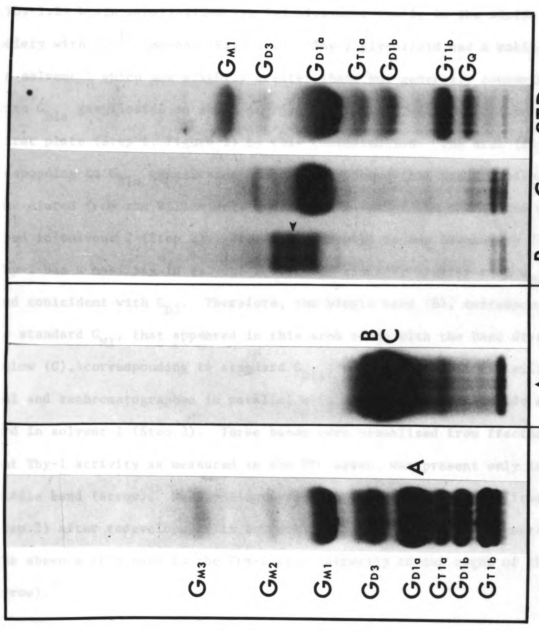
GM3
GM2
GM1
GD3
GD1a
GT1a
GD1b
GT1b
A
B
C
GM1
GD3
GD1a
GT1a
GD1b
GT1b
GQ
Step 1
Step 2
A
B
C
STD
Step 3

Figure 2.

present in mouse brain. We therefore undertook to visualize and purify Thy-1 by combining the sensitivity of autoradiographic procedures with the sequential use of solvents 1 and 2.

Thy-1 glycolipid was visualized by autoradiography of ICR Swiss (Thy-1.2) brain gangliosides labeled biosynthetically in the sialic acid moiety with [1-^{14}C]ManNAc (Figure 2). Thy-1 glycolipid had a mobility in solvent 1 which was slightly greater than, but generally contaminated with G_{D1a} ganglioside as shown in Figure 1. Thy-1 was observed on the first plate (Step 1, Figure 2) by this contamination. The area (A), corresponding to G_{D1a} ganglioside and slightly above (but not including G_{D3}), was eluted from the silica gel, spotted on a second TLC plate, and developed in solvent 2 (Step 2). From previous work in our laboratory (121), Thy-1 has a mobility in solvent 2 which is slightly greater than G_{M1} and conicident with G_{D3}. Therefore, the single band (B), corresponding to standard G_{M1}, that appeared in this area along with the band directly below (C), corresponding to standard G_{D1a}, were eluted from the silica gel and rechromatographed in parallel with mouse brain ganglioside standard in solvent 1 (Step 3). Three bands were visualized from fraction B but Thy-1 activity as measured in the PFC assay, was present only in the middle band (arrow). No Thy-1 activity was detected in band C (from Step 2) after redevelopment in solvent 1 (Step 3). This is supported by the absence of a band in the Thy-1 area (directly to the right of the arrow).

The use of sequential solvents was effective for separating ganglioside mixtures containing Thy-1 as shown in Figure 2. These solvent systems were applied to a simpler two dimensional TLC procedure in order to achieve separation in a shorter amount of time. AKR/J (Thy-1.1) or

Figure 3. Autoradiograms of ManNAc Labeled Brain Gangliosides. Gangliosides were labeled by intracranial injection of [1-^{14}C]ManNAc and were separated in two dimensions with solvents 1 (vertically) and solvent 2 (horizontally). The assayed fractions are labeled with numbers which correspond to the PFC assay results in Figure 4. Fraction 6 refers to the area surrounding all the assayed spots.

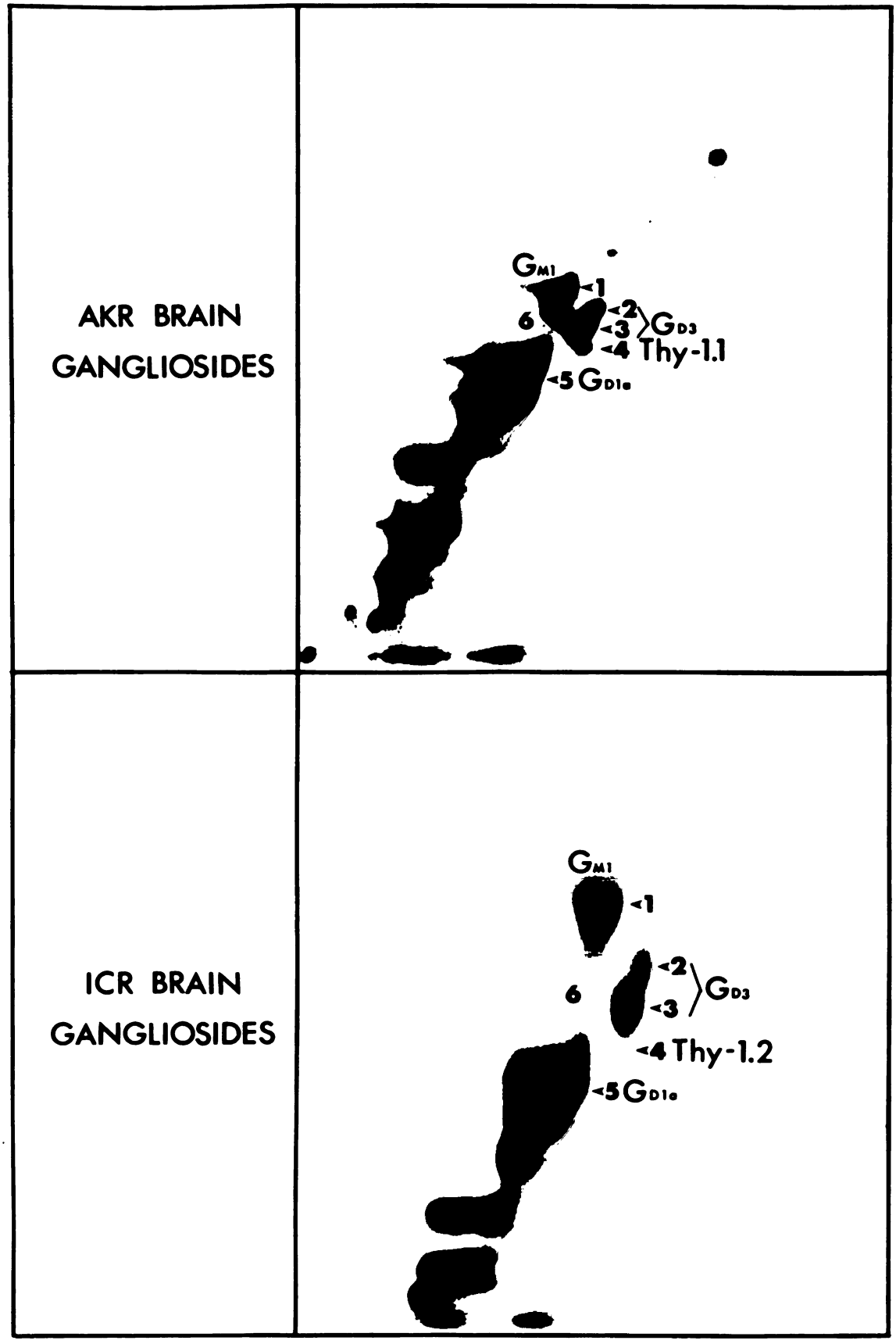
AKR BRAIN
GANGLIOSIDES
GM1
1
2
3
6
GD3
4 Thy-1.1
5 GD1a
ICR BRAIN
GANGLIOSIDES
GM1
1
2
3
6
GD3
4 Thy-1.2
5 GD1a

Figure 3.

Figure 4. Anti-Thy-1 PFC Assay of Brain Gangliosides. The gangliosides assayed refer to the numbers in Figure 3. The values shown are the mean ± standard error of five cultures. Application of the Students t test to the standard errors for the samples results in p values less than 0.05 when compared to the Thy-1 active fraction. Positive control for the anti-Thy-1.2 PFC assay was a column G_{M1} fraction containing Thy-1 glycolipid.

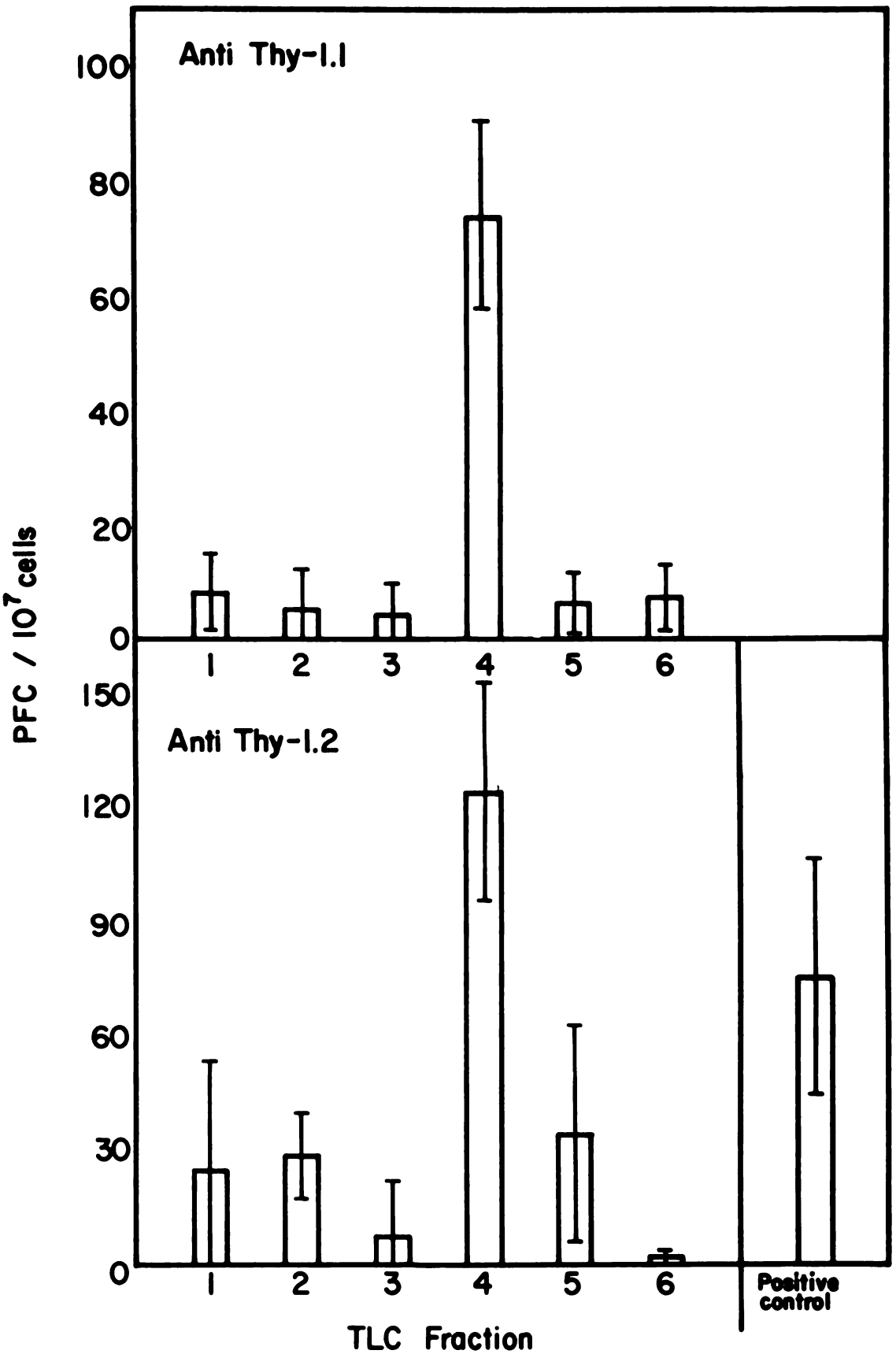
Anti Thy-1.1
100
80
60
40
20
0
1
2
3
4
5
6
PFC / 10^7 cells
Anti Thy-1.2
150
120
90
60
30
0
1
2
3
4
5
6
Positive control
TLC Fraction

Figure 4.

ICR Swiss (Thy-1.2) brain gangliosides, biosynthetically labeled with [1-^{14}C]ManNAc, were applied in one spot in the corner of TLC plates. These plates were developed twice in solvent 2 on one axis, then developed once in solvent 1 on the second axis. A number of spots were detected by autoradiography of these plates (Figure 3). These spots were identified by relative TLC mobility compared to the ganglioside standards on the side of the plates (not shown in Figure 3). The AKR brain gangliosides were tested with the anti-Thy-1.1 PFC assay and the ICR brain gangliosides were tested with the anti-Thy-1.2 PFC (Figure 4). The Thy-1 antigenicity tests revealed one Thy-1.1 glycolipid (Fraction 4) from AKR brain and one Thy-1.2 glycolipid (Fraction 4) from ICR brain. The TLC fraction numbers in Figure 4 correspond to the numbers on the autoradiographs in Figure 3. The positive control was Thy-1.2 glycolipid isolated by one dimensional TLC from C3H brain.

Thy-1 activity was associated with the spot directly below G_{D3} and to the right of G_{D1a} in the orientation shown (Figure 3). Brain Thy-1.1 glycolipid (Fraction 4) consistently migrated slightly faster in solvent 1 and 2 than Thy-1.2 glycolipid (Fraction 4). Using G_{D3} as a reference, Thy-1.1 was further to the right and below G_{D3} than Thy-1.2, which was directly below G_{D3} (Figure 3). When Thy-1 glycolipids were eluted from these plates and rechromatographed by one dimensional TLC no contamination with other gangliosides was detected.

Thy-1 antigen is expressed on certain lymphoma cell lines and we wished to determine if Thy-1 glycolipids were present on these cells. BW5147 (Thy-1.1) and S49.1 (Thy-1.2) lymphoma cell lines were labeled biosynthetically with [1-^{14}C]palmitic acid or [1-^{14}C]ManNAc. Glycolipids from these cells were isolated using the extraction and two-dimensional

Figure 5. Autoradiograms of Palmitate Labeled Lymphoma Cell Glycolipids. Chromatography was performed as described in Figure 3. The assayed fractions are labeled with numbers which correspond to the anti-Thy-1 PFC assay in Figure 6. The number 10 refers to the area surrounding the spots which were assayed.

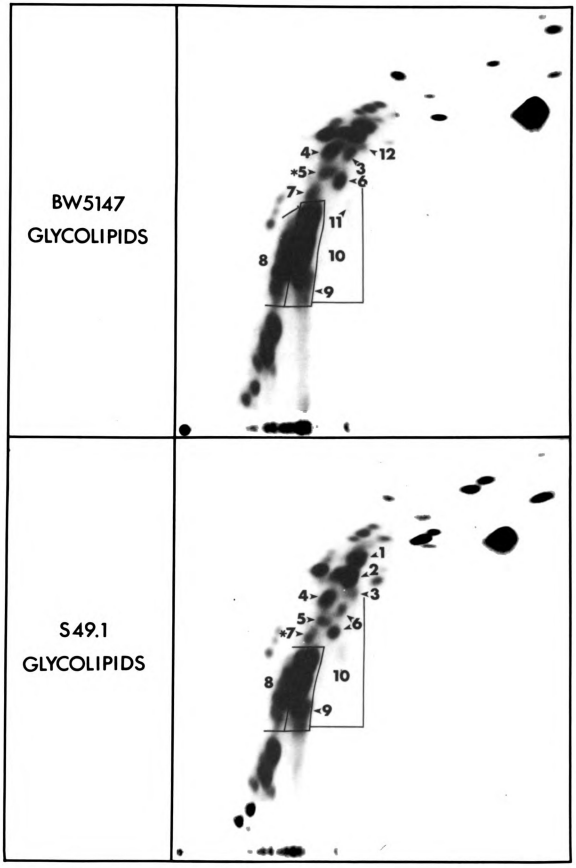
BW5147
GLYCOLIPIDS
4
12
*5
3
6
7
11
8
10
9
S49.1
GLYCOLIPIDS
1
2
4
3
5
6
*7
8
10
9

Figure 5.

Figure 6. Anti-Thy-1 PFC Assay of Lymphoma Glycolipids. The glycolipids assayed refer to the numbers in Figure 5. The values shown are the mean ± standard error of five cultures. Application of the Students t test to the standard errors for these samples results in p values less that 0.05 when compared to the Thy-1 active fraction. Positive controls are brain Thy-1 glycolipids.

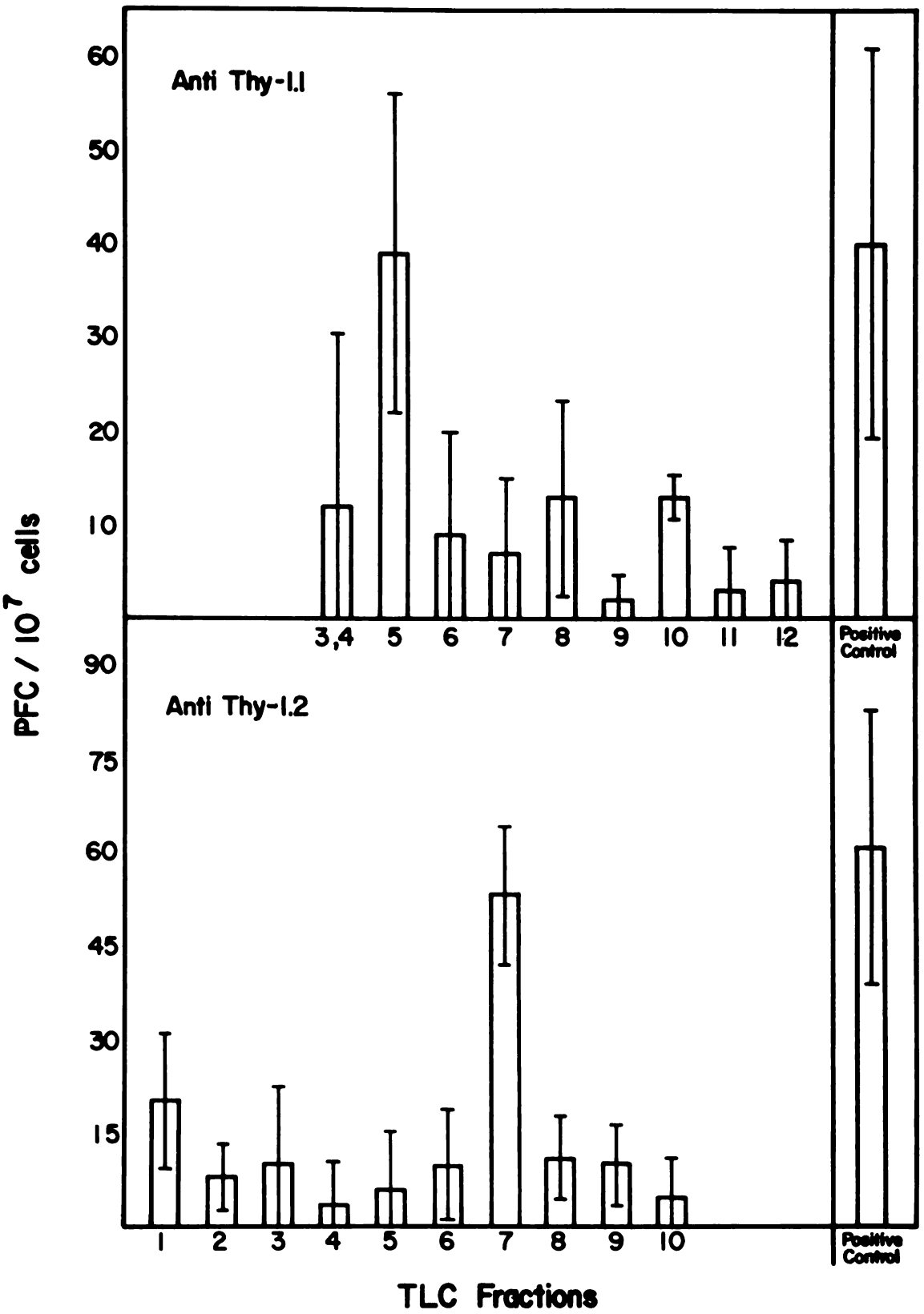
Anti Thy-1.1
Anti Thy-1.2
PFC / 10^7 cells
60
50
40
30
20
10
90
75
60
45
30
15
3,4
5
6
7
8
9
10
11
12
1
2
3
4
5
6
7
8
9
10
Positive Control
Positive Control
TLC Fractions

Figure 6.

Figure 7. Autoradiogram of ManNAc Labeled Lymphoma Cells. Lymphoma cells were labeled with [1-^{14}C]ManNAc in culture. Chromatography was performed as in Figure 3. Thy-1 glycolipids are indicated in this figure.

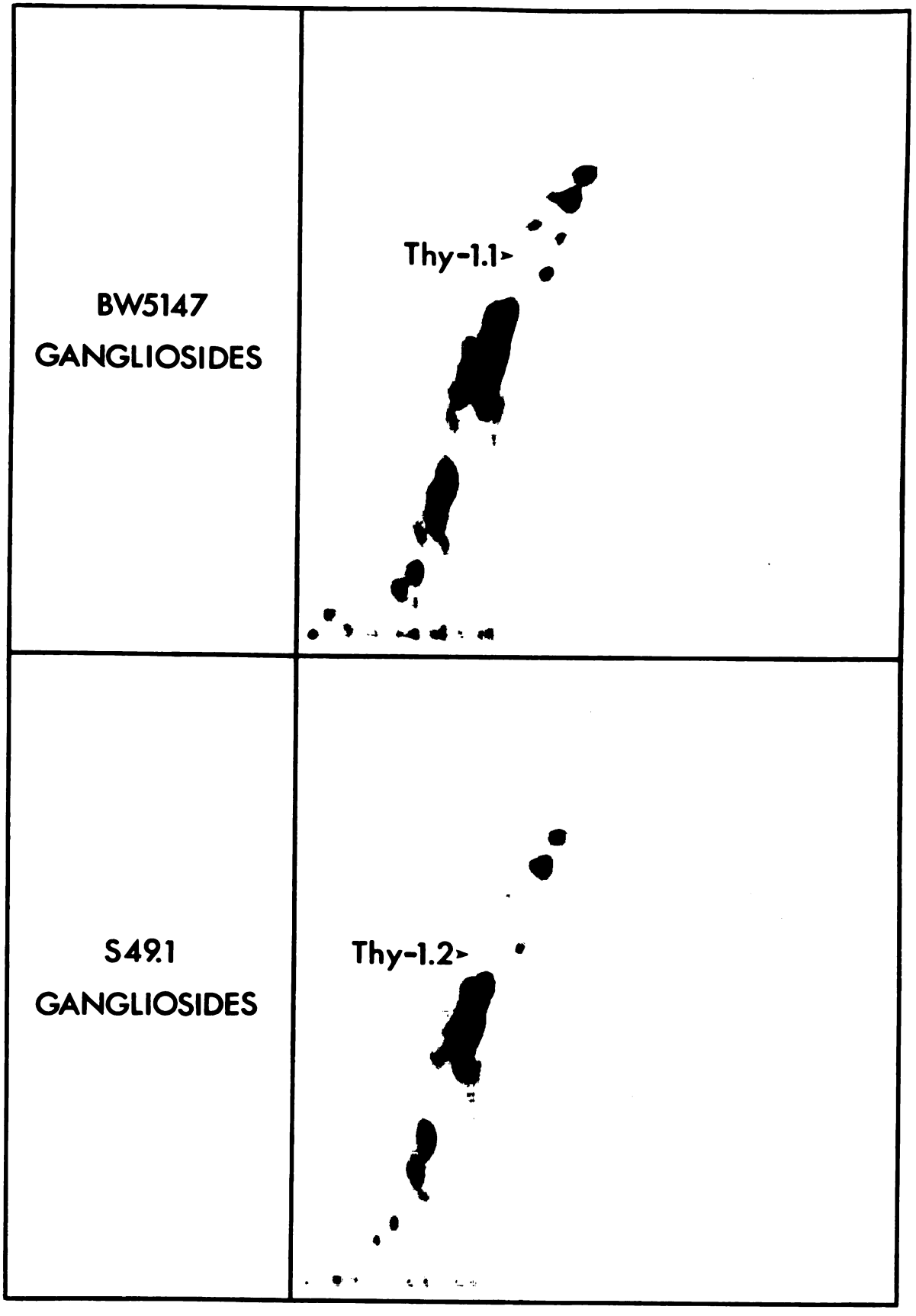

Figure 7.

TLC procedures mentioned above. A number of spots were revealed by autoradiography of [1-^{14}C]palmitic acid labeled glycolipids (Figure 5). Spots tested for Thy-1 activity were selected by comparison to brain ganglioside standards on the side of the TLC plate. BW5147 glycolipids were tested with the anti-Thy-1.1 PFC assay and S49.1 glycolipids were tested with the anti-Thy-1.2 PFC assay. One Thy-1.1 glycolipid was detected from BW5147 cells (Fraction 5, Figure 6) and one Thy-1.2 glycolipid from S49.1 cells (Fraction 7, Figure 6). The fractions in Figure 6 correspond to the numbered spots on the autoradiography in Figure 5.

All cellular glycosphingolipids should be labeled with [1-^{14}C]palmitic acid but gangliosides were selectively labeled with [1-^{14}C]ManNAc. BW5147 and S49.1 lymphoma cell lines were labeled in culture with [1-^{14}C] ManNAc. Autoradiography of these labeled gangliosides from two dimensional TLC plates is shown in Figure 7. Most of the glycolipids labeled with palmitate in Figure 5 in the lower left quadrant of the plate were labeled with ManNAc. Many of the other spots in the upper right quadrant (Figure 5) were neutral glycolipids and hence were not labeled with ManNAc. Lymphoma Thy-1.1 and Thy-1.2 were both labeled with ManNAc as seen in Figure 7.

Mild acid and neuraminidase treatment and DEAE cellulose chromatography were used to further characterize the Thy-1 active compounds. Short neuraminidase treatment and mild acid conditions, which result in the removal of sialic acid, destroyed the anti-Thy-1 PFC response to these antigens (Table I). Furthermore both Thy-1.1 and Thy-1.2 glycolipids bound to DEAE cellulose, confirming their acidic nature.

Thy-1 glycolipids isolated using two-dimensional TLC from brain (AKR/J and ICR) and lymphoma cells (BW5147 and S49.1) were tested for

TABLE I

PROPERTIES OF THY-1 GLYCOLIPIDS

GLYCOLIPID	THY-1.1	THY-1.2
Treatment	Anti-Thy-1.1 Response[a]	Anti-Thy-1.2 Response[a]
None	51 ± 18	63 ± 6
DEAE:bound	36 ± 5	45 ± 9
:not bound	8 ± 6	1 ± 1
C.p. Nase [b]	13 ± 6	15 ± 9
HCl[c]	1 ± 1	7 ± 2

[a]The values shown are the mean ± standard error of five cultures.

[b]*Clostridium perfringens* neuraminidase was incubated with glycolipids for 1 hr at 37°C.

[c]0.1N HCl was incubated with glycolipids for 30 min at 80°C.

Figure 8. Anti-Thy-1 PFC Assay for Allogeneic Specificity of Brain and Lymphoma Thy-1 Glycolipids. Thy-1 glycolipids isolated from the two dimensional TLC plates in Figures 3 and 5 were tested for allogenic specificity with the anti-Thy-1 PFC assays. The values shown are the mean $\pm$ standard error of five cultures. Application of the Students t test to the standard errors for these samples results in p values less than 0.05 when compared to the Thy-1 active fraction.

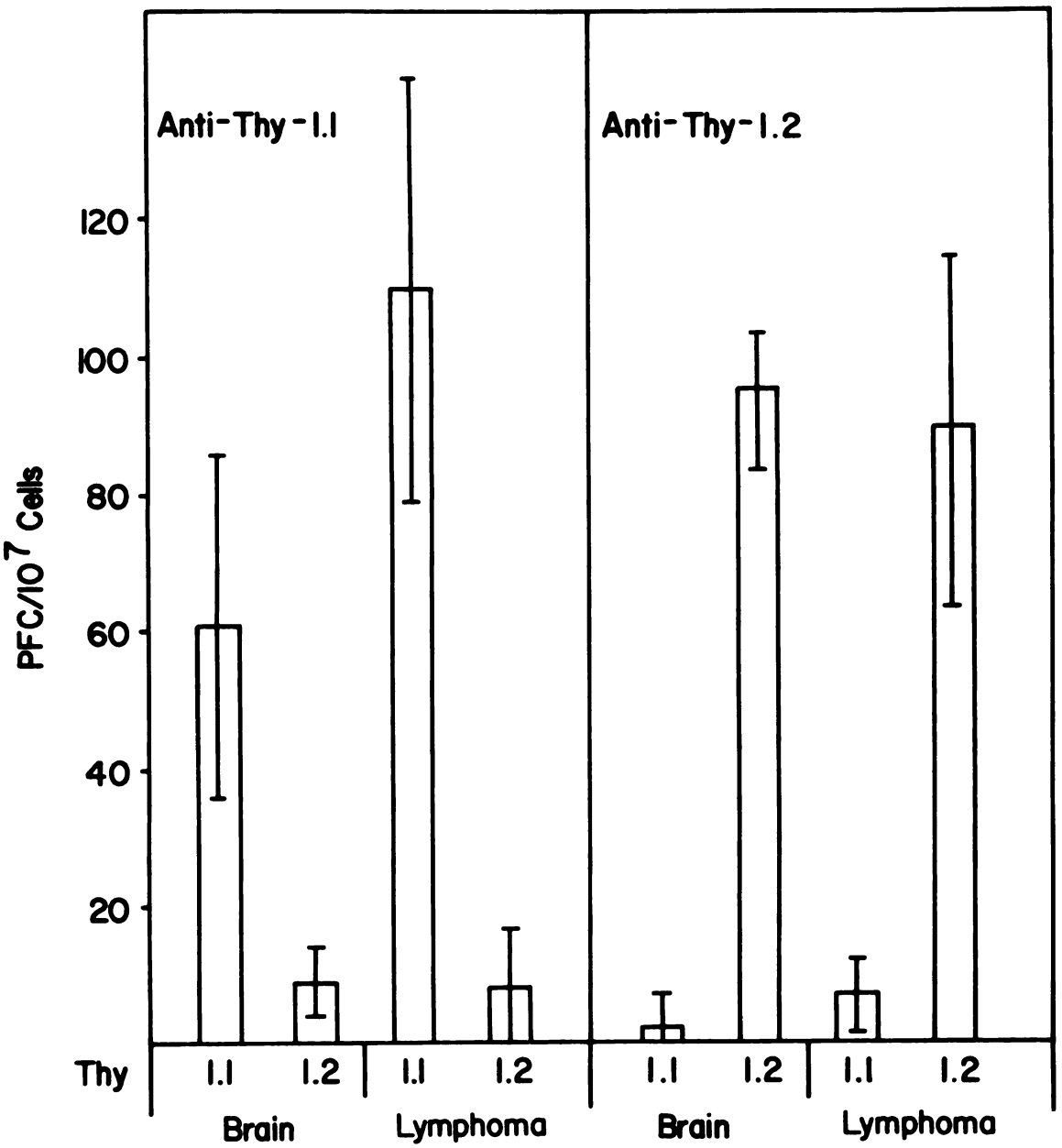
Anti-Thy-1.1
Anti-Thy-1.2
PFC/10^7 Cells
120
100
80
60
40
20
Thy
1.1
1.2
1.1
1.2
1.1
1.2
1.1
1.2
Brain
Lymphoma
Brain
Lymphoma

Figure 8.

allogenic specificity with the anti-Thy-1 PFC assay. All four samples were tested in both the anti-Thy-1.1 and anti-Thy-1.2 PFC assays. AKR/J brain and BW5147 glycolipids (both of allotype Thy-1.1) elicited a response only in the anti-Thy-1.1 PFC assay (Figure 8). Thy-1 glycolipids of the Thy-1.2 allotype showed no response in this assay. ICR brain and S49.1 glycolipids (both of allotype Thy-1.2) elicited a response only in the anti-Thy-1.2 PFC assay but Thy-1 glycolipids of the Thy-1.1 allotype did not.

DISCUSSION

We have previously reported on the occurence of glycolipids from mouse brain and thymocytes which carry Thy-1 antigenicity and specificity (121). Other researchers have reported on the isolation of Thy-1 active glycoproteins from rat (115) and murine (116) lymphoma cells. The immune response assay we have used recognized both of these antigen types as well as demonstrating their allogeneic specificity (121). Precedent for similar carbohydrate determinants on glycoprotein and glycolipid antigens is found in the ABO, Lewis blood group systems (49,50) as well as in the report of Hakomori and Tonegawa (144) on the nature of "ganglioproteins" - that is those proteins which share antigenic moieties with ganglioside (glycolipids with sialic acid). We now report the isolation, partial purification and radiolabeling of Thy-1 glycolipids from neonatal mouse brain and from two lymphoma cell lines.

Thy-1 glycolipids were detected in fractions eluted from thin layer plates, but even though the active compounds migrated differently in different solvent systems, they could not be visualized by conventional thin layer sprays. This was due to the relatively small amounts of Thy-1 glycolipids present. This problem was overcome by radiolabeling

the glycolipids with different radioactive precursors followed by two dimensional TLC and autoradiography. Gangliosides were labeled by incubation with [1-^{14}C]ManNAc which is a precursor of sialic acid and which has been previously shown by neuraminidase hydrolysis to be incorporated exclusively into the sialic acid moieties of gangliosides (19). [1-^{14}C] palmitate acid was used as a precursor for labeling glycolipids because palmitate is incorporated into sphingosine by condensation with serine and it may also be added as the acyl group of ceramide (16-18). Phospholipids and triglycerides were eliminated from our preparations by mild base hydrolysis and dialysis (162).

Radiolabeled Thy-1 glycolipid was isolated from ICR mouse brain using sequential solvents in one dimensional TLC (Figure 2). Autoradiography of these TLC plates, identified for the first time, a glycolipid band with Thy-1 activity (Figure 2, Step 3). The band (arrow) identified as Thy-1 in Step 3 was accompanied by two contaminating gangliosides even after two TLC runs in different solvents. A more effective procedure for the isolation of Thy-1 glycolipid was developed using two dimensional chromatography in which a single plate was developed in both solvents and autoradiography was used to detect the location of radiolabeled glycolipids. Thy-1 was separated from other gangliosides as shown in the autoradiograms of the two dimensional TLC plates in Figure 3. Thy-1 glycolipids were eluted from these two dimensional plates and rechromatographed by one dimensional TLC. No contamination was detected in the autoradiogram of this plate demonstrating the effectiveness of this procedure.

Thy-1 glycolipids had different TLC R_f values in solvent 1 than solvent 2. The mobility of Thy-1 glycolipids (Figure 3) was very similar

to G_{D3} ganglioside in that they migrated ahead of G_{M1} in solvent 2, and behind G_{M1} and slightly ahead of G_{D1a} in solvent 1. We have also found that Thy-1.1 migrates slightly faster than Thy-1.2 in solvent system 1 (Figure 1). Structural differences between Thy-1.1 and 1.2 presumably result in different mobility in either solvent system.

Some lymphoma cell lines express Thy-1 on their cell surfaces. These cultured cells may be grown in large quantities and serve as a good source of membrane components. We found these lymphoma cells to be a good *in vitro* source of Thy-1 glycolipid which could be labeled with lipid precursors. Lymphoma cell glycolipids were labeled with either [1-^{14}C]palmitate or [1-^{14}C]ManNAc. About 30 compounds were labeled with both palmitate and ManNAc in these cell lines (Figure 5 and 7). Additional compounds labeled with palmitate (Figure 5) were probably neutral glycolipids because they were resistant to mild base treatment.

Several lines of evidence indicate that Thy-1 glycolipids are gangliosides. The glycolipids were isolated in ganglioside fractions after Folch partition and thin layer chromatography and they were resistant to mild base treatment which results in the hydrolysis of other lipids. The Thy-1 glycolipids were labeled with ManNAc and palmitic acid indicating the presence of sialic acid and sphingosine respectively. Ion exchange chromatography with DEAE cellulose indicated the acidic nature of glycolipids. In addition to ion exchange chromatography, the presence of sialic acid on the active molecule was indicated by hydrolysis with neuraminidase. Both Thy-1.1 and Thy-1.2 active glycolipids were destroyed by one hour treatment with this enzyme. This does not necessarily prove however, that sialic acid was part of the antigenic determinant

because this treatment may have rendered the molecule inactive due to decreased solubility.

The Thy-1 activity of the glycolipids from brain and lymphoma cells was studied by comparing the Thy-1 allotypic specificity of the antigens. These results (Figure 8) indicated that the antigen displayed only the allotype of the mouse strain from which they were isolated. Thus, the AKR strain and the BW5147 cell line were positive in the anti-Thy-1.1 PFC assay and the ICR strain and the S49.1 cell line were positive in the anti-Thy-1.2 PFC assay. No crossreaction was observed at the levels tested. Demonstration of the reciprocal allogeneic specificity is important because this supports the suggestion that the glycolipids carry specificities which parallel the serological specificity of the Thy-1 allotypes. This finding excludes the possibility that the anti-Thy-1 PFC assay recognizes other cell surface alloantigens, such as Ly 8 (165).

The Thy-1 glycolipids isolated from brain and lymphoma cells were very similar in the thin layer mobility properties. In solvent 1 both brain and lymphoma Thy-1.1 glycolipid migrated faster than the Thy-1.2 glycolipid and in solvent 2 the Thy-1.1 glycolipid migrated only slightly faster than the Thy-1.2 glycolipid. Thus it appears that the brain and lymphoma antigens were similar in thin layer chromatographic mobilities and in their immunological specificities.

In summary, identification of the minute quantities of Thy-1 glycolipids in brain and lymphoma cells was possible by incorporation of radiolabeled lipid precursors, palmitate and ManNAc. These compounds were separated from contaminating glycolipids by two dimensional TLC, visualized by autoradiography and tested for Thy-1 activity with the anti-Thy-1 PFC assay. The demonstration of one active compound from each cell source

with Thy-1 allogeneic specificity parallels the serological properties of these antigens. The ganglioside nature of these compounds is supported by DEAE cellulose chromatography, neuraminidase treatments and labeling with the sialic acid precursor ManNAc. We support the contention that Thy-1 antigenicity is carried in mice on ganglioside and glycoprotein structures.

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