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IMMUNOCHEMISTRY OF THY-1 ANTIGENIC DETERMINANTS: CHARACTERIZATION WITH NEURAMINIDASE AND MONOCLONAL ANTIBODIES

By

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ABSTRACT

IMMUNOCHEMISTRY OF THY-1 ANTIGENIC DETERMINANTS:
CHARACTERIZATION WITH NEURAMINIDASE AND MONOCLONAL ANTIBODIES

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The nature of mouse Thy-1 antigens has been studied. Neuraminidase (Nase) treatment of purified Thy-1 glycoproteins, THy-1 glycolipids or shed membrane complex which contains both molecules, has decreased the expression of Thy-1 antigenicity as shown by an anti-THy-1 plaque forming cell assay and a direct binding radioimmunoassay. Three different sources of Nase were used and results were identical. Kinetic studies have shown that 50% decrease of antigenicity was achieved by approximately 6 to 9 hours of Nase treatment. Nase treatment of thymocyte surface glycoproteins with radiolabeled sialic acid demonstrated that sialic acids were uniformally removed from glycoproteins including Thy-1 and several glycoproteins of relative molecular mass (Mr) of 120,000 Da to 45,000 Da, but extensive hydrolysis was achieved by 12 to 18 hours

incubation of NP-40 cell lysate.

The expression of Thy-1.2 antigenic determinants as recognized by a xenogeneic (clone 30-H12) and an allogeneic (clone HO-13.4-2.2) monoclonal antibodies was studies. Both antibodies precipitated glycoproteins of indistinguishable Mr from NP-40 lysate of iodinated thymocytes. After Nase treatment the quantity of alloantibody precipitate was reduced but the xenoantibody precipitate was unchanged suggesting a difference in recognition of Thy-1.2 molecules. The Mr of the precipitate was unchanged after Nase treatment. NEPHGE analysis of the Nase treated NP-40 lysate exhibited only two components. Both Nase products were precipitable with the xenogeneic anti-Thy-1.2 but only the most basic component was precipitable with allogeneic anti-Thy-1.2. The presence of two distinct Thy-1.2 bearing glycoproteins was shown by NEPHGE analysis of immunoprecipitates. These antibodies each precipiated a distinct family of components of a very basic nature and similar charge hterogeneity. The componets differed in their overall pI with the allogeneic antibody precipitating the most basic components. The difference in pI was verified by the mild acid treatment which resolved the allogeneic precipate in one major very basic component and the xenogeneic precipitation into a more acidic component.

DEDICATED TO
JUDITH

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INTRODUCTION

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treatment although this may have been due to the reduced activity of glycopeptides compared to intact protein. Carbohydrates have also been suggested to be factors in the Thy-1 determinant.

Mouse lymphocyte Thy-1 glycoprotein (also designated T25) has an apparent Mr of 25 to 30,000 Da and has been found to be heterogenous with respect to isoelectric point. Using a two-dimensional gel electrophoresis system Ledbetter et al. demonstrated that Thy-1 glycoproteins from mouse thymocytes were resolved into a family of closely related glycoproteins of 25,000 to 30,000 Da with extensive charge and size heterogeneity. Neuraminidase (Nase) treatment of the thymocytes followed by pH analysis by gradient electrophoresis indicated a decrease in the heterogenity of Thy-1 glycoproteins, suggesting that the observed charge variations were caused by sialic acid. Hoessli et al. reached a similar conclusion by using the same gel elctrophoresis system and also observed different charge heterogeneity of Thy-1 between thymocytes and lymph node cells.

Glycolipids isolated from murine lymphoma lines, brain, and thymocytes which express the Thy-1.1 or 1.2 specificity have the properties of gangliosides, in that they contain sialic acid. Experiments described here were designed to examine the chemical nature of Thy-1 antigenic determinants by Nase treatment and be reaction with monoclonal anti-Thy-1

antibodies. Sensitivity to Nase treatment was assessed by treating the antigens with Nase followed by measurement of remaining Thy-1 by the Thy-1 immune response-PFC assay and by a direct immunoradiometric assay. Results of these experimental approaches are presented in the first of three articles (published in Molecular Immunology, 17:1389). Furthermore, Nase treatment and immunoprecipitation with two monoclonal antibodies have been used to characterize and distinguish two Thy-1.2 specificities carried on two different glycoproteins. Results are present in the second article (submitted for publication). The third article (submitted for publication) focuses on a chemical modification of membrane sialic acid for identification of Thy-1 glycoproteins and a direct approach to study the kinetics of Nase treatment on thymocyte Thy-1 glycoproteins. In the appendix, efforts to identify Thy-1 antigeneic determinants on shed complexes and glycolipids are also presented. The establishment of a radioimmunoassay for the detection of Thy-1 antigens on membrane complexes of thymocytes is presented in article 4 (published in Molecular Immunology, 17:1381). The identfication and characterization of Thy-1 glycolipids of murine brain and lymphoma cell lines using a two-dimensional thin-layer chromatography system is presented in Article 5 (published in J. Immunol.,123:1977).

LITERATURE REVIEW

I. INTRODUCTION.

Thy-1 antigen was first described by Reif and Allen ( ) in the thymus and brain of C3H and AKR mice. The tissue distribution in the immune system of the mouse is generally restricted to thymocytes and thymus-derived T lymphocytes and is related to the maturation and differentiation of functional T lymphocytes. Antigens similar to Thy-1 (or cross reactive with Thy-1 have been identified in lymphoid, brain and epidermal tissue of the rat, dog, and human but the cellular distribution in these species, especially in lymphocytes appears different from that of mouse. Thy-1 antigenic determinants have been proposed to exist on two types of glycoconjugates, glycolipid and glycoprotein. Review of prevous works in the biochemistry of Thy-1 antigens is covered in Section II. Review of biochemistry of glycolipids had been covered elsewhere. Section III reviews the biochemistry of lymphocyte membrane glycoproteins and general methods to study cell surface glycoproteins. Glycoproteins are generally categorized by the linkage structures between peptide and carbohydrate, so Section IV covers the characteristics of carbohydrate sturctures especially sugar moieties involved in the linkage structure in glycoproteins.

II. THY-1 ANTIGEN.

Mouse Thy-1 active glycolipids.

Esselman and Miller reported that a glycolipid preparation of mouse brain was able to inhibit the cytotoxicity of anti-Thy-1 antiserum. This observation has suggested the existence of lipid forms of Thy-1 antigen, analogous to the existence of glycolipid forms of the blood group substances in human (1). When the glycolipid preparation was further separated on TLC, the Thy-1 activity was demonstrated in GM_1, enriched fractions. The lipid forms of Thy-1 antigen have also been demonstrated by a plaque forming cell (PFC) assay which measured the immune response against Thy-1 antigen directly (2). Brain glycolipids and thymocyte glycolipids were found to induce specific PFC's when cultured with spleen cells from animals of a different Thy-1 phenotype (Thy-1.1 or Thy-1.2). For instance, AKR brain gangliosides (Thy-1.1) cultured with C3H (Thy-1.2) spleen cells induced anti-Thy-1.1 immune response and the antibody secreting B cells could be enumerated in a lawn of AKR target thymocytes. The formation of individual blue plaque indicated the presence of a anti-Thy-1 antibody producing B cells. Results from these studies showed that gangliosides prepared from brain tissue or thymocytes induced an antibody response against Thy-1 antigen and the responses were specific for Thy-1. The specificity of the response was also shown by absorption experiments. The antigen was treated with allogenic anti-Thy-1 antisera

Thy-1 antisera before culturing with spleen cells. It was shown that 1) anti-Thy-1.1 antiserum blocked Thy-1.1 glycolipids in the induction of anti-Thy-1 PFC response and did not block Thy-1.2 glycolipids; and 2) anti-Thy-1.2 antiserum only blocked Thy-1.2 glycolipids in the induction of Anti-Thy-1 PFC response.

To determine the relationship between the glycoprotein form and glycolipid form of Thy-1 antigens, the glycoprotein was prepared from brain tissue by detergent solubilization and lentil lectin column chromatography, and tested in anti-Thy-1 PFC assay. Similar to the glycolipid form, Thy-1 glycoprotein was also capable of inducing specific anti-Thy-1 immune response (3). This suggested that Thy-1 glycoprotein and Thy-1 glycolipid were serologically similar. There are two possibilities for the existence of these two forms of Thy-1 antigen: 1) they are cross-reacting antigens, due to complete or partial similarity in antigeneic determinants; or 2) there are multiple specificities in the immune responses against Thy-1 antigens, elicited by glycolipid and glycoprotein separately. The chemical analysis of structures and the production of monoclonal antibodies will help distinguishing between Thy-1 glycolipid and Thy-1 glycoprotein.

Brain Thy-1 glycolipid was separated from major cell surface gangliosides such as G_{M1}, GD_{1a}, G_{D3}, by using thin layer chromatography (TLC) developed with chloroform:

methanol: H_2O and chloroform: methanol: NH_4OH solvents sequentially (4). This indicated that the structures of Thy-1 glycolipids were different from those of the known glycolipids. Mouse thymocyte Thy-1 glycolipids were also separated in the same TLC system and had similar motility as the brain Thy-1 glycolipids, indicating that they might be identical in structure and composition. Another approach to purify Thy-1 glycolipids is the two dimensional TLC (4). Gangliosides of brain or lymphoma cells were first radiolabeled with precursor compounds such as (^3H)-N-acetyl-mannosamine or (^{14}C)-palmatate, the gangliosides were then separated on TLC plates which were developed with chloroform: methanol: H_2O in one dimension and with chloroform: methanol: NH_4OH at a second dimension. Components were identified by autoradiography, recovered, and tested in anti-Thy-1 PFC assay to identify Thy-1 glycolipid. Major gangliosides were identified by comparison with standards and by gas-liquid chromatography. Only one component among more than twenty gangliosides tested showed Thy-1 antigenicity. Autoradiographs of the TLC plate showed that this component was physically separated from other components including G_{M1}, GD_{1a}, and G_{D3}.

The biosynthetical radiolabeling data also suggested that, similar to major gangliosides, the Thy-1 glycolipids were readily radiolabeled with palmatate and N-acetyl-

mannosamine which are precursors for sphingosine and N-acetylneuraminic acid respectively. It is most likely that Thy-1 glycolipids have same ceramide backbone and the different TLC motility of this compound is due to structural variation in the covalently-linked carbohydrate moieties.

In addition to the evidence obtained from the radiolabeling of Thy-1 glycolipids, there are several lines of evidence to show that Thy-1 glycolipids contain sialic acid residues. Neuraminidase treatment of (^{3}H) sialic acid radiolabeled Thy-1 glycolipids completely removed the radioactivity from Thy-1 glycolipids, as judged by the disappearance of radioactivity from the Thy-1 glycolipid spot on TLC. Similarly mild acid hydrolysis completely removed the radioactivity from Thy-1 glycolipids. Thy-1 glycolipids have also been shown to be eluted from DEAE-Sepharose column along with disialogangliosides, suggesting that each Thy-1 glycolipid contained two sialic acid residues (5). Asialo-Thy-1 glycolipids failed to elicit any significant anti-Thy-1 immune response when tested in anti-Thy-1 PFC assay. This suggests that sialic acid residues are essential for the expression of Thy-1 antigenicity (6).

Rat Thy-1.1 antigen.

Rat Thy-1 antigen was identified with an xenoantiserum prepared by immunization of rabbits with rat brain homogenate or rat brain glycoprotein (7). Three injections of 1mg lentil lectin column purified glycoprotein was used

over several months. After thorough absorption with rat liver homogenate the antiserum was shwon to be specific for determinants carried on rat brain and thymus. Evidence suggested that determinants recognized by this antiserum might be on the same molecule as the determinant recognized by allogenic mouse anti-Thy-1.1 serum. Rat-specific determinants were also recognized by this antiserum. No sequential immunoprecipitation has yet been carried out to determine if this antiserum contains antibodies specific for antigens beside Thy-1.

The tissue distribution of Thy-1 antigen in rat differsr in several aspects from that of the mouse. The highest concentrations of Thy-1 antigen were identified in both brain and thymus (which was similar to mouse) but Thy-1 positive cells were also found in bone marrow. Goldschneide et al. (9,10) have shown that Thy-1 positive bone marrow cells (obtained by use of Fluorescence Activated Cell Sorter (FACS)) were pluripotent hemopoietic stem cells which formed colonies in spleen of irradiated syngenic rats (CFU-S). Treatment of bone marrow cells with antiserum plus complement removed this population of CFU-S. No decrease of CFU-S was observed when mouse bone marrow cells were treated similarly. It seems probable that Thy-1 expression in the rat does not require the thymic environment. Only 3% to 7% of rat peripheral lymphocytes have Thy-1 antigen, in contrast to 35% to 60% Thy-1 positive cells in mouse

peripheral lymphoid tissues. This suggests a different role for rat Thy-1 antigen on rat thymocytes and T lymphocytes.

Biochemistry of rat Thy-1.1 glycoprotein.

A glycoprotein containing Thy-1 determinants has been purified from rat brain and thymus (11). Preliminary chemical analysis showed that the purified glycoprotein preparation contained about 30% carbohydrate. Thy-1 Glycoproteins from rat thymocytes could be separated on the basis of binding with lentil lectin into bound and unbound fractions. Chemical analysis of the two fractions showed that the amino acid compositions were similar but differences were observed in carbohydrate compositions. Therefore Thy-1 antigen determinants were identified on species of glycoproteins of apparent molecular weight of 25,000 daltons, with basically homogeneous amino acid sequence but heterogeneous carbohydrate compositions.

The amino acid sequence of rat brain Thy-1 glycoprotein has been reported in detail (12). The purified glycoprotein (from lentil lectin column) was further purified on a affinity column containing monoclonal anti-Thy-1.1 antibody (clone M.R.C. OX7). The purified glycoprotein was then reduced and alkylated before protease digestion with trypsin or V8 protease. The sequence was determined from tryptic or V8 protease peptides by manual dansyl-Edman degradation and automatic amino acid sequencer. The whole sequence contains 111 amino acids with 3 possible sites of carbohydrate

linkage. The basic features of the rat Thy-1 are as follows: 1) the amino terminus is blocked and consisted of a pyroglutamic acid; 2) no extended sequence of hydrophobic amino acids was found which is characteristic of many cell surface glycoproteins; 3) two disulfide were identified, Cys9-Cys111 and Cys19-Cys85; 4) three locations of carbohydrate attachment, Asn-23, Asn-74, and Asn-98; and 5) the C-terminus residue has not been directly identified but Cys-111 is the last conventional amino acid. The C-terminus containing tryptic peptide or V8 protease peptide could be readily recovered in a detergent micelle form whereas without detergent C-terminus peptide could only be recovered in a highly aggregated form. This indicates the presence of a lipophilic sequence at the C-terminus which probably is responsible for the insertion of Thy-1 antigen into the membrane. No structural evidence of this lipophilic moiety has been reported as yet.

Mouse brain Thy-1.2 glycoprotein.

Letarte et al. (13) have reported the purification of Thy-1.2 glycoprotein from B10 mouse brain. Procedures for purification of this glycoprotein were essentially the same as described for the purification of rat Thy-1 glycoprotein; i.e. detergent solubilization, gel filtration and lentil lectin chromatography. The glycoprotein had a molecular weight of 26,000 to 29,000 daltons as determined by SDS-electrophoresis. The antigenicity was determined by the

absorption of purified glycoprotein with several sources of antisera including a rabbit anti-rat Thy-1 glycoprotein serum, a rabbit anti-mouse Thy-1 glycoprotein serum, and an AKR anti-C3H antiserum. The rabbit anti-mouse Thy-1 glycoprotein serum was prepared by immunizing rabbits with purified mouse brain Thy-1 glycoprotein. This antiserum has been shown to be specific for both AKR and C3H thymocytes and Wistar rat thymocytes. Purified mouse brain Thy-1 glycoprotein was able to completely remove the cytotoxic activity against B10 thymocytes of rabbit anti-rat brain Thy-1 glycoprotein but only removed 60% of the cytotoxicity from AKR anti-C3H antiserum. In the direct binding assay using ^{125}I labeled antiserum, purified Thy-1 glycoprotein failed to block binding of labeled AKR anti-C3H antiserum to B10 thymocytes whereas effective blocking was observed when labeled rabbit anti-rat brain Thy-1 glycoprotein antiserum was used. Therefore the authors concluded that the purified Thy-1 glycoprotein contained the xenogeneic Thy-1 determinant(s) (recognized by the rabbit antiserum), whereas the allogeneic determinant was not unequivocally demonstrated. We have demonstrated that this purified mouse brain Thy-1 glycoprotein induced specific anti-Thy-1.2 plaque forming cell responses (5).

In a recent report, Letarte et al. (14) have used a monoclonal anti-Thy-1.2 antibody (clone F7D5, obtained from Lake, P. (15)) to demonstrate the Thy-1.2 allogenic

determinant on the purified mouse brain Thy-1 glycoproteins. In the absorption experiments of either cytotoxicity assay or direct binding assay, purified Thy-1 glycoprotein in the dose range of 1 ug to 10 ug effectively removed the activity of the monoclonal antibody against B10 thymocytes. A similar degree of absorption could be obtained by pretreatment with 10^7 to 10^8 B10 thymocytes. Therefore, by using higher concentration (100 fold increase of Thy-1 glycoprotein, (compared to previously reported dose of 10 ng to 100 ng), the allogenic Thy-1 determinants were demonstrated on the purified mouse brain Thy-1 glycoprotein.

They have also demonstrated that the AKR anti-C3H antiserum contained anti-MuLV specificity. DBA/2 thymocytes were shown to effectively remove higher than 90% cytotoxic antibody from AKR anti-C3H serum at the level of 10^5 cells whereas 10^7 to 10^8 Friend leukemia cells were necessary to obtain a similar absorption. Pretreatment of ^{125}I labeled antiserum with DBA/2 thymocytes removed 40% of binding to glutaraldehyde-fixed Friend cells. To determine the type of viral antigen recognized by AKR-anti-C3H antiserum, disrupted Gross passage A MuLV or Rauscher MuLV were used to absorb the antiserum and residual antibody activity was assayed by binding to Friend cells or DBA/2 thymocytes. About 80% of antibody activity against Friend cells and 60% activity against DBA/2 thymocytes was absorbed by Gross passage A MuLV whereas Rauscher MuLV was unable to remove

the activity against either type of cells. Therefore the authors concluded that in the previous report, only marginal absorption of AKR anti-C3H serum observed with purified Thy-1 glycoprotein was due to the contaminating anti-viral antibody.

Thy-1 glycoproteins of lymphoma cells.

Trowbridge et al. (20) reported the identification of Thy-1 antigen on BW5147 lymphoma cells. Cell membrane molecules were iodinated with lactoperoxidase (39) and immunoprecipitation of detergent solubilized, ^{125}I labeled membrane glycoproteins with rabbit anti-thymocyte serum produced several major labeled glycoproteins including T-200 and T-25 (Thy-1). Zwerner, et al. (21) have also reported purification of Thy-1.1 glycoprotein from BW5147 cells. The purified glycoprotein had the molecular weight of 25,000 daltons as determined by SDS-gel electrophoresis. The xenogenic determinant was readily detected on purified glycoprotein with rabbit antiserum whereas allogenic determinant of Thy-1 antigen was only demonstrated with 100 fold greater amounts of antigen.

The selections and genetics of Thy-1 negative lymphoma mutants.

Murine anti-Thy-1 antisera and complement were used to select Thy-1 negative mutants (24). Stable mutant clones were selected and maintained by periodic recloning. Most Thy-1 negative mutants contain less than 1% surface Thy-1

antigen as compared with parental lines. The explanation for the origin of these cell surface antigen mutants may be genetic or epigenetic. Genetic alterations may include regulatory and structural gene changes and epigenetic events are most likely changes in cell membrane processing leading to failure to incorporate Thy-1 antigen. Complementation analysis between two Thy-1.2 negative mutants and between mutants and parent lines have indicated that, 1) the failure to express Thy-1.2 is recessive because fusion between mutant and parent lines generated Thy-1.2 positive cells; and 2) the changes in mutants are most likely genetic because the fusion between the two mutants reexpressed Thy-1 antigen on the cell surface (25). Thy-1 negative mutants also appear spontaneously as shown by Hyman and Stallings (25). They have used an enrichment procedure and an indirect immuno-selection method for selection of Thy-1 negative mutant in the absence of antibody and complement. Limited numbers of parent cells were added to each petri dish of a series of dishes (100 cells/dish). After the cells had grown, a portion of cells were removed and the percentage of antibody and complement resistant cells was determined. The culture with highest percentage was chosen for further enrichment. After 7 cycles, the best dish contained 8,658 resistant cells per 10^5 cells (about 1 in 12 cells was resistant). The cells were cloned and 30 clones were picked, one showed resistance to anti-Thy-1 antiserum.

could cause the loss of Thy-1 on the cell surface.

Biochemical analysis of Thy-1 negative mutants.

Trowbridge and Hyman (26) characterized the genetic defects by using methods including cell surface radiolabeling, immunoprecipitation and SDS-gel electrophoresis. Iodinated glycoproteins detected by autoradiography showed that T25 was missing in all four mutants tested but was detected on the autoradiograph of the iodinated proteins from each of the Thy-1 positive lymphomas. No detectable differences in other iodinated proteins beside T25 were observed between each variant and its respective parental cell line. Immunoprecipitation with anti-Thy-1 antiserum of iodinated proteins of mutant lymphomas also confirmed the absence of iodinated T25 in the mutant lymphomas. Iodinated hybrid cells derived from fusions between pairs of complementary or noncomplementary Thy-1 negative mutants were similarly characterized and T25 appeared only on hybrid cells of complementary pairs but not on noncomplementary pairs. Therefore these results have confirmed the genetic studies of Thy-1 negative mutants that T25 was absent from the surface of negative mutants and only reappeared on hybrid cells derived from pairs of complementary mutants.

Absence of T25 on the surface of Thy-1 negative mutants raised the question as to whether T25 was synthesized by the variants. Biosynthetical radiolabeling of variants with ^{3}H-

mannose and ^{3}H-leucine and analysis by immunoprecipitation and SDS-gel electrophoresis have demonstrated that antigenically cross-reacting molecules were synthesized by the variants except the variant of class D. T25 glycoproteins made by class A, B, C, and E mutants differed from the wild-type molecule in its electrophoretic mobility. On the basis of their motilities, the T25 glycoproteins of Class A, B, and C mutants appeared to be 500 - 1500 daltons smaller than the wild-type T25 glycoprotein and in the class E mutant T25 molecule migrated as a species about 4000-5000 daltons smaller. Immunofluorescence staining of live cells with a rabbit anti-T25 antiserum has confirmed the absence of T25 on the surface of mutants (27). Using frozen sections examined by immunoflourescence, the parental cells showed staining predominantly on the cell membrane with areas of weak diffuse cytoplasmic fluorescence. In contrast, the variants of class A, B, C, and E showed only diffuse patchy fluorescence throughout the cytoplasm. Similar patterns of T25 distribution were obtained when parent and mutant cells were examined with ferritin-conjugated antiserum and immunoelectron microscopy. These results suggest that the structural differences of T25 glycoproteins in mutants are a consequence of mutations in genes beside structural gene coding for T25 polypeptide (except class D). It has been suggested that the genetic defects in these mutants affect

the post-translational processing of the T25 glycoprotein, preventing T25 glycoprotein from reaching the cell surface (26).

This interpretation was further supported by results from chemical analysis of the oligosaccharide chains of T25 glycopeptides synthesized by Thy-1 negative mutants (28,29,31). T25 glycoproteins may contain two types of asparagine-linked oligosaccharide chains, the complex-type containing NeuAc, galactose, glucosamine, and mannose, and the simple-type containing only glucosamine and mannose (12,73). Trowbridge et al, have reported that pronase digestion of T25 glycoproteins radiolabeled with either ^{3}H-mannose or ^{3}H-glucosamine followed by chromatography on a Bio-gel P-6- column separated glycopeptides containing the two types of oligosaccharide chains (28). The smaller molecular weight peak contained only radioactive mannose or glucosamine, suggesting that it had simple-type oligosaccharide. The larger glycopeptide contained radioactive mannose, glucosamine, or galactose, suggesting that it has complex-type oligosaccharide chains. When the T25 glycoproteins of mutant class A or C were digested with pronase and chromatographed on a Bio-gel P-6 column, the larger glycopeptide peak was absent and the small glycopeptide had identical M.W. as that of wild type T25 glycopeptide with simple-type oligosaccharide chain. In class E mutant, not only were the high molecular weight galactose-rich glycopeptides missing, but the glycopeptides

of the mutant glycoprotein containing only mannose and glucosamine were smaller than those of the wild-type molecules. The oligosaccharide chains of T200 glycoproteins in wild type and variants were similarly studied and no differences were observed, indicating that differences in glycosylation in variant cells were restricted to T25 glycoproteins (29).

Defects of glycosylation in T25 glycoproteins do not in all cases prevent these molecules from reaching the cell surface. When a series of lectin-resistant lymphoma cell lines were characterized for cell surface antigens and for the pattern of glycosylation in T25 and T200 glycoproteins, two different patterns were obtained (30). In the PHA-resistant lymphoma cell lines, cell surface concentrations of Thy-1, H-2, and GP69,71 were unaltered but the electrophoretic motility of Thy-1 and GP69,71 was greater than wild-type molecules in two variants, in the other two variants, the electrophoretic mobility remained the same. In ConA-resistant lymphoma cell lines, these mutants were deficient in membrane Thy-1 antigen although their expression of H-2 and GP69,71 was similar to wild-type cells. The autoradiograph of the iodinated cell surface glycoproteins of ConA-resistant mutants showed that the iodinated band corresponding to Thy-1 glycoproteins was not detected. Both groups of mutants have been shown to have defects in glycosylation. Chromatographs of T25

glycopeptides from galactose, mannose or glucosamine radiolabeled PHA-resistant mutants on Biogel P-6 column indicated that the larger molecular weight galactose-rich glycopeptide peak was greatly reduced and the smaller glycopeptide peak was unaltered, as compared to wild-type T25 glycopeptides. Chromatographs of radiolabeled T25 glycopeptides from ConA-resistant mutants showed a similar pattern to the class E Thy-1 negative mutant that the larger glycopeptide peak was missing and the smaller glycopeptide with simple-type oligosaccharide chains was of lower molecular weight than that of wild-type glycopeptides. Therefore contrary to defects in [previously mentioned Thy-1 negative variants, the defects in PHA-resistant mutants do not prevent the expression of Thy-1 antigen on cell surface, but defects in ConA-resistant mutants have similar effects as defects of Thy-1 negative mutants.

Anti-Thy-1 monoclonal antibodies.

Marshak-Rothstein et al. (22) reported the production of anti-Thy-1 monoclonal antibodies specific for either Thy-1.1 or Thy-1.2 antigen. Anti-Thy-1.1 specificity was obtained from fusion of myeloma cells with (Balb/c X Balb.k) F_1 spleen cells immunized with AKR/J thymocytes. Anti-Thy-1.2 specificity was obtained by fusion with AKR/J spleen cells immunized with C3H thymocytes. The antibodies were cytotoxic for 95% of thymocytes of the appropriate Thy-1 phenotype; 60% to 70% lymph node cells; and 35% spleen

cells. The estimated titer was as high as one million, in comparison to one thousand for conventional antiserum. Functional studies have shown that the monoclonal antibodies plus complement would eliminate thymocyte and T-cell dependent responses and leave the B-cell dependent response such as the lipopolysaccharide (LPS) mitogenic response intact. Pretreatment of B10 cytotoxic T lymphocytes(CTL) with monoclonal antibody plus complement also eliminated CTL response. Based on these observations, it was concluded that the specificity was restricted to Thy-1.

Similar approaches were used in several laboratories to obtain monoclonal anti-Thy-1 antibody. A rat anti-mouse Thy-1.2 antibody was reported by Ledbetter et al (23). ^{125}I labeled mouse thymocyte glycoprotein immunoprecipitated by this anti-Thy-1.2 antibody (clone 30-H12) was analyzed by two dimensional gel electrophoresis. It consisted of a family of glycoproteins of 25,000 to 30,000 daltons with heterogeneity of size and charge. Neuraminidase treatment of glycoproteins suggested that the charge variation was caused by variable amounts of terminal sialic acid on the glycoproteins. The size differences have been suggested due to various degrees of glycosylation as in the case of rat thymus glycoproteins (11).

Lake et al. (15) also reported production of monoclonal anti-Thy-1 antibodies specific for Thy-1.2 antigen of mouse thymocytes and for Thy-1.1 antigen of rat brain cells.

Functional analysis of these antibodies have shown that, in contrast to anti-Thy-1.2 antibodies, the anti-Thy-1.1 antibody failed to remove CTL precursor cells in the presence of complement but removed CTL responder cells effectively. Analysis by FACS of in vitro cultured AKR spleen cells showed that the Thy-1.1 determinant recognized by monoclonal anti-Thy-1.1 antibody appeared on the cell surface during the five-day culture period. The anti-Thy-1.2 antibody was capable of eliminating both precursor-CTL and CTL. This suggests that a subset of Thy-1 antigenicity (related to rat brain Thy-1.1 antigenicity) was acquired during the activation process of mouse cytotoxic T lymphocytes, but absent on precursor CTL.

Human homologue of Thy-1 glycoprotein.

Using the human T lymphoblastoid cell line MOLT-3, Ades et al. (16) reported the identification and purification of Thy-1 cross-reacting antigen. The procedures for purification were identical to those used for other Thy-1 glycoproteins. This glycoprotein was able to absorb cytotoxic antibody from either rabbit anti-rat brain Thy-1 or the congenic anti-Thy-1 antiserum. The rabbit antiserum raised against this purified protein was cytotoxic to MOLT-3 cells and AKR/J thymocytes. This rabbit antiserum also precipitated a protein of 25,000 to 28,000 daltons from human peripheral T lymphocytes (E-rosette positive cells). Goat anti-mouse brain Thy-1 antiserum also precipitated a

protein of 25,000 to 28,000 daltons from human peripheral T lymphocytes. Therefore, this suggests that human Thy-1 antigen has cross-reactive determinants with mouse Thy-1 antigen.

A similar molecular weight glycoprotein was isolated from human brain. Antigenicity was assayed by the inhibition of complement-mediated cytotoxicity against CBA (Thy-1.2) thymocytes with a rabbit anti-human brain antiserum (17). The purification procedure was similar but wheat germ lectin chromatography and DEAE-Sepharose chromatography were also used. Thy-1 antigen was found in both wheat germ lectin bound and unbound fractions which indicated that the purified glycoprotein was heterogeneous in degree of sialylation. After storage, dimerization of Thy-1 glycoprotein occured which was still antigenically active. Attempts to clarify the nature of Thy-1 antigenic determinants have shown that: 1) the heat treatment did not affect the antigenicity in contrast to reports by A. Williams (11); 2) neuraminidase treatment had no effect but periodate treatment (20mM, 6hr) destroyed all activity; 3) reducing agents such as cysteine and dithiothreitol also removed the antigenicity and half of activity was lost in the presence of 0.1% SDS; and 4) reconstitution of detergent solubilized Thy-1 glycoprotein in liposome form increased Thy-1 antigenicity. Therefore, this suggests that tertiary structures and intact

carbohydrate moieties are necessary for expression of Thy-1 antigenicity. A monoclonal anti-human brain Thy-1 glycoprotein antibody has been reported recently. (18) The antibody was shown to be specific for a molecule of approximately 25,000 daltons. Immunofluorescence studies with cell sorter have confirmed that Thy-1 is absent from human peripheral lymphocytes and cells from lymphoid organs. Only 7% thymocytes showed positive staining. Immunofluorescence staining of lymphoid tissue sections showed an unusual location of Thy-1 positive cells: bright staining was restricted mainly to the periphery of the thymus lobule; the marginal zone, and some periarteriolar lymphocytes in the spleen; and the post-capillary venules of lymph node. Therefore, human Thy-1 antigen has very different tissue distribution from those found in rat and mouse. Human Thy-1 antigen has been suggested to be related to recirculation of lymphocytes and a marker for early T lymphocytes in man. (19)

III. LYMPHOCYTE MEMBRANE GLYCOPROTEINS.

Biochemistry of cell surface antigens.

In the murine system, some 40 to 50 different antigens had been reported (32). Among them, several antigens have been characterized in detail. The biochemical nature of several important molecules are discussed below.

H-2K and H-2D gene products are glycoprotein in nature, with Mr of 45 to 50,000 daltons as determined by SDS-gel electrophoresis (33,34). They contain either one or two carbohydrate chains of 3,300 daltons covalently linked to the peptide backbone. The antigenicity of H-2 molecules are resided in amino acid sequence variations (33). Gene product of Ia-1 locus (I-A subregion) have been characterized (32). Immunoprecipitation experiments have identified a 58,000 to 60,000 daltons molecule consisting of two chains of 33,000Da and 27,000Da. No detailed sequence data has yet been reported. Analysis of Ly-1.1 specificity by cell surface iodination, immunoprecipitation, and analysis of SDS-gel electrophoresis showed Lyt-1.1 antigen to be a glycoprotein of molecular weight of 67,000 daltons (35). This glycoprotein has been shown to be trypsin-resistant. Durda, et. al. have purified molecules carrying Ly-2/Ly-3 specificities by similar approaches (36). Results indicated that they were glycoproteins of molecular weight of 35,000 daltons. Ly-2 and Ly-3 specificities have been thought to be carried on either the same molecule or on different closely associated molecules. Sequential immunoprecipitation experiments have shown that Ly-2.1 and Ly-3.1 specificities were precipitated independently after NP-40 solubilization of the membrane. Ly-3.1 antigen has been shown to be more labile to trypsin digestion than Ly-2.1.

Radiolabeling and gel electrophoresis of lymphocyte membrane glycoproteins.

Membrane glycoproteins and proteins have been studied by radiolabeling techniques such as neuraminidase/galactoxidase treatment followed by NaB^3H_4 reduction (37), or periodate-NaB^3H_4 treatment (38), and by lactoperoxidase-catalysed iodination (39). The radiolabeled molecules are then separated, on the basis of size and charge, by SDS-gel electrophoresis and two dimensional 2-D gel electrophoresis (40,41,42). Additional information about the nature of individual glycoproteins are obtained by immunoprecipitation with specific antisera or by lectin binding (32). Potential differentiation antigen could be discovered if purified cell populations such as T lymphocytes and B lymphocytes were radiolabeled and gel patterns of radiolabeled cell surface molecules were compared. Glycoproteins found restricted to one or more populations may suggest a possible relationship with differentiation.

Analysis by two-dimensional gel electrophoresis (2-D) provides great resolution of membrane glycoproteins and proteins. Hoessli et al. (43) have shown that at least twenty components were resolved on 2-D maps from radioiodinated lymphocytes. The molecular weights of these components range from higher than 2 million daltons to 10,000 daltons. At least five components were heterogeneous with respect to pI and molecular weight. In the first

dimension, proteins were separated on an equilibrium pH gradient of 4.8-7.6 or on nonequilibrium pH gradient to separate components on the basis of pI. Variations in charge were in some glycoproteins possibly due to difference in the degree of sialylation on the molecule because neuraminidase treatment of intact cells appeared to reduce the number of variants found in at least two components (100,000Da and 27,000Da). When the two-dimensional maps of thymocytes and lymph node cells were compared, potential differentiation-related antigen could be identified. Radioiodinated components of 150,000, 140,000 and 110,000Da were on Balb/c thymocytes but were missing from lymph node cells. Maps of radioiodinated lymph node cells showed two characteristic components of 190,000 and 60,000Da which were missing from maps of thymocytes. The complexity of membrane glycoproteins was reduced if these molecules were further characterized on the basis of the antigenicity, lectin binding, and the ability of different radiolabeling techniques to radiolabel these components.

Lactoperoxidase-catalyzed iodination. In the presence of hydrogen peroxide, iodide and a nucleophilic receptor, a number of peroxidases will catalyze the formation of a carbon-halogen bond (39). Lactoperoxidase is commonly used because of its optimal activity under physiological conditions. When intact red blood cells were radioiodinated with this method, 96% of radioactivity incorporated was

found in cell membrane and less than 4% radioactivity was found in hemoglobin. The main iodinated amino acid residue was determined by thin layer chromatography as monoiodotyrosine. The presence of great excess of (ratio 10^5) chloride ion will inhibit the iodination to about 50%.

Periodate-tritiated borohydride. Labeling of sialic acid residues on glycoproteins was first described by Lenten and Ashwell (44). The two terminal exocyclic carbon atoms of sialic acid were selectively cleaved upon exposure to periodate with no destruction of the ring structure or cleavage of the glycosialic bond. Tritiated borohydride reduction of the aldehyde group resulted in a stable radioactive derivative of the original glycoprotein. Under optimal condition, periodate oxidation of orosomucoid (79) consumes 2 moles of periodate per mole of sialic acid with the release of 1 mole of formaldehyde. The radioactive derivative was obtained from glycoprotein by acid hydrolysis (80% recovery) or by enzyme digestion (60% recovery) and it had the same Rf as synthetic 7-carbon neuraminic acid (heptulosonic acid derivative) judged by paper chromatography. Gahmberg and Anderson using a similar method reported that membrane glycoproteins of intact red blood cells were efficiently radiolabeled. Under mild conditions (1 to 5 mM periodate, for 20 minutes at 4 C°) higher than 90% of the radioactivity was bound to membrane. Between 60% to 70% membrane bound radioactivity was released

by mild acid hydrolysis (0.1M H_2SO_4, 80°C, 1hr). Extensive periodate oxidation of glycoproteins results in destruction of terminal, nonreducing residues as well as neutral sugars linked to the oligosaccharide chain in which carbon 3 is unsubstituted (55). Extensive periodate treatment has been used on structure determination and is the basis of the widely used Smith degradation. The mild periodate/boro-hydride method has also been used to label mouse lymphocytes and other type of cells (45,46,48).

Biosynthetic labeling with radioactive metabolic precursors.

(^{35}S)- methionine, [^{14}C]CMP-sialic acid, and [^{3}H] or [^{14}C]-mannose are among some of precursors most commonly used to biosynthetically label membrane glycoproteins. Although a high efficiency of incorporation of isotope into membrane is usually obtained with biosynthetic labeling, radiolabeling is not restricted to membrane molecules. Therefore clean membrane preparation are required to obtain radiolabeled membrane glycoproteins. Labeling with [^{14}C]CMP-sialic acid, has been used successfully to locate sialoglycoproteins and sialyltransferases to the luminal side of the Golgi apparatus in mouse and rat liver cells (47).

Galactose oxidase-tritiated borohydride.

Galactose oxidase oxidizes terminal galactosyl and N-acetylgalactosaminyl residues forming the corresponding C6

aldehydes. The aldehyde residues are reduced with tritiated borohydride to introduce tritium onto glycoproteins (37). The galactose oxidase does not penetrate cell membrane therefore resulting in labeling of surface glycoproteins selectively. Sialic acids are often linked to penultimate galactosyl residues and, therefore, more efficient labeling is achieved by pretreating the cells with neuraminidase. Treatment with neuraminidase followed by treatment with galactose oxidase permits identification of sialylgalactosyl and sialyl-N-acetylgalactosaminyl residues on cell surface if the labeling pattern is compared to that obtained without prior neuraminidase treatment. Both glycoproteins and glycolipids are radiolabeled by this method. The common procedure involves incubation of 10^7 to 10^8 cells in PBS with 0.05IU Vibrio cholera Neuraminidase for 30 minutes at 37 C. Galactose oxidase in PBS is added and incubated for 3 hours at 37 C. After washing, cells are reduced with 1mCi tritiated sodium borohydride for 30 minutes and then washed thoroughly (37).

Immunoprecipitation of radiolabeled glycoproteins.

Precipitation of radiolabeled membrane glycoproteins with xeno- or allo- antisera identifies components which are unique to the target cell types. Hoessli et al (43) have reported that anti-mouse lymphoma antiserum identified 12 to 13 distinct components in immunoprecipitate from radioiodinated thymocyte or lymph node cell lysates. Three

weakly radioiodinated components were concentrated, including glycoproteins of 100,000 and 50,000 to 70,000Da. No new differences were observed between thymocyte and lymph node cells, which might have escaped detection from 2-D analysis of whole cell lysates. Similar approaches were used by Kamarck and Gottlieb (49) to identify changes of cell surface glycoproteins and proteins during fetal thymus maturation and differentiation. Immunoprecipitation with rabbit anti-mouse thymocyte serum showed that: 1) radioiodinated glycoproteins of fetal thymocytes on day 15 of gestation contained very little T25 (Thy-1 antigen) as did thymocytes of day 19 of gestation; 2) T200 (200,000Da) was present in both adult thymocytes and fetal thymocytes of day 15 and 19 of gestation but fetal thymocytes contained one larger glycoprotein (higher than 200,000Da) not present on adult thymocytes. Other differences were observed but not characterized.

T200 of adult thymocytes was first reported by Hyman and Trowbridge et al. (80). Radioiodination of BW5147 lymphoma cells followed by immunoprecipitation of NP-40 solubilized membrane glycoproteins with rabbit anti-mouse thymocyte serum precipitated two major iodinated glycoproteins, T200 and T25. T200 had the apparent molecular weight of 200,000 daltons (actually between 170,000 and 190,000Da). It has been shown to be a major plasma membrane component of thymocytes and thymus-derived

lymphocytes. Omary and Trowbridge (50) have reported that two distinct regions of the molecule, the part exposed to the cytoplasmic side of the plasma membrane and the part exposed to the outer side, could be defined by radiolabeling methods in combination with protease treatment, immunoprecipitation, and peptide mapping. A relatively protease-resistant domain, which was exposed on the cell surface and contained the antigenic site recognized by a monoclonal anti-T200 antibody, contained most of the mannose-oligosaccharide units of the glycoprotein and all of the amino acid residues labeled by lactoperoxidase-catalyzed iodination of intact viable cells. The remaining portion of the molecule contained a region which was exposed on the cytoplasmic side of plasma membrane A ^{125}I-labeled tryptic peptide derived from this region was obtained only when membrane preparations from disrupted cells were radioiodinated. This region contained a phosphoserine residue which was labeled with $^{32}PO_4$ in vivo and was also relatively more trypsin labile.

Identification of T25 from rat was unsuccessful due to the fact the rat Thy-1 antigen was not labeled with ^{125}I (46). Techniques used to radiolabel the carbohydrate moieties of glycoprotein on intact cells have been used instead to successfully identify T25 in rat (46). Rat lymphoid cells were oxidized with 1mM $NaIO_4$ in terminal sialic acid and the resulting aldehyde groups were reduced

with NaB^3H_4, as described by Gahmberg and Anderson (37). Labeled cells were solubilized and radiolabeled glycoproteins were characterized by SDS-gel electrophoresis and immunoprecipitation. From rat thymocytes, at least eight heavily labeled glycoproteins were identified and fewer bands were identified from T-lymphocytes or B-lymphocytes. T-lymphocytes contained heavily labeled glycoproteins of Mr 170,000 Da and 85,000 Da and B-lymphocyte contained a heavily labeled glycoprotein of higher than 200,000 Da and a few other weakly labeled glycoproteins. Immunoprecipitation of thymocyte glycoproteins with rabbit anti-mouse thymocyte serum, anti-leukocyte common antigen (LC) (clone MRCOXI) and anti-rat thymocyte antibody (clone w3/13) precipitated glycoproteins of 25,000, 150,000, and 95,000 Da. MRCOX1 antibody also precipitated a glycoprotein of 170,000Da from T-lymphocytes and a glycoprotein of 200,000Da from B-lymphocytes. The lymphocytes. The similarity between T200 of mouse thymocytes and LC antigens of rat thymocytes has not yet been clarified (51).

Lectin and glycoprotein interactions.

The carbohydrate moieties of glycoproteins are the basis for interactions with lectins. The sugar specificities of individual lectins have been well established. For example, Concanavalin A (ConA) is specific for D-Mannose and D-Glucose; Helix pomatia (snail)

hemaglutinin (HP) is specific for N-acetyl-D-galactosamine, and Lentil lectin (LC) has the same specificities as ConA. Structures of oligosaccharides to which these sugar moieties are linked also determines the affinity of interaction with lectin, for example Krusius et al. (53) have shown that glycopeptides with a biantennary structure were bound by ConA whereas glycopeptides with a triantennary structure were not (see next section).

Interaction of lectins and membrane glycoproteins have been investigated. Five to seven major radioiodinated glycoproteins from human T-lymphocytes or B-Lymphocytes have been shown to bind strongly to lentil lectin (LC) and PHA. (molecular weights ranging from 200,000 to 25,000 Da (52)). One component of 35,000 to 37,000 Da was identified from B-lymphocytes but not from T-lymphocytes whereas T-lymphocytes have characteristic 25,000Da component which is absent from B-lymphocytes. From mouse thymocytes, T25 (Thy-1) and T200 glycoproteins have been shown to bind to a ConA-Sepharose column (11), although Thy-1 antigenicity was also identified in ConA unbound fractions. This is due to the heterogeneity of carbohydrate moieties in Thy-1 glycoproteins (54). The 150,000 and 25,000 Da glycoproteins identified in rat thymocytes by $NaIO_4/NaB^3H_4$ radiolabeling and immunoprecipitation, were bound to ConA affinity column whereas 95,000Da glycoprotein was not (46).

IV. GLYCOPROTEIN CARBOHYDRATE STRUCTURE AND FUNCTION.

Characteristics of carbohydrate linkages of glycoproteins.

Glycoproteins have a native composition in which carbohydrate moieties are covalently linked to a polypeptide backbone (55-58). Glycoproteins have a wide distribution in nature and serve a vast number of functions. There are glycoprotein enzymes and hormones; glycoproteins are found in blood and secretions, in cell membranes, and in connective tissue. The sugars that commonly occur in glycoproteins include galactose (Gal), mannose (Man), glucose (Glu), N-acetylglucosamine (GlcNac), N-acetylgalactosamine (GalNac), sialic acid (NeuAc), fucose (Fuc) and xylose (Xyl).

During the past decade new analytical methods such as gas-liquid chromatography or stepwise degradation of oligosaccharide chains by specific glycosidases have been devised that does not require large quantities of sample. Thy-1 active glycoproteins have been shown to contain large content of carbohydrate moeities (about 30%). Neuraminidase treatment of Thy-1 have shown decrease in expression of antigenicity suggesting the important of carbohydrate units in Thy-1 expression. The characteristics of the structures of glycoprotein bound carbohydrate units are reviewed here in order to elucidate the possible interactions of carbohydrate moieties to polypeptide backbone.

Glycoproteins can be distinguished on the basis of the

sugar and amino acid involved in the linkage between carbohydrate and peptide moieties. This section covers three types of saccharide-protein linkage from various biological sources: 1) the N-Acetylglucosaminyl-asparagine linkage found in plasma glycoproteins; 2) the N-acetylgalactosaminyl-serine/threonine linkage found in epithelium secretory glycoproteins; and 3) the galactosyl-hydroxylysine linkage found in collagen-like substances.

Glycopeptides with N-Acetylglucosaminylasparagine linkage.

The N-glycosidic linkage between N-acetylglucosamine and asparagine was first characterized in the early 1960's and has been subsequently described in a variety of plasma glycoproteins including fetuin, transferrin, the immunoglobulins, glycoprotein hormones, thyroglobulin, and in erythrocyte membrane glycoproteins (59-63). The bond was established by partial acid hydrolysis of glycopeptides and analysis of components containing both carbohydrate and aspartic acid. The linkage structure was found by comparison to synthetic compounds to be 2-acetamido-L-aspartamido-1,2-dideoxy-beta-D-glucosamine. In more recent studies, glycopeptides containing asparagine as the only amino acid were usually prepared by extensive pronase treatment of the purified glycoprotein. Removal of sugar residues which permited isolation of the linkage moiety was accomplished by sequential exoglycosidase treatment, sequential Smith degradation, and partial acid hydrolysis.

An approach involved using endo-beta-N-acetylglucosaminidases which were capable of cleaving the di-N-acetylchitobiose unit (64,65). These endo-glucosaminidases are specific to their substrate so that some glycoproteins may not be susceptible to their action.

Cleavage of the N-glycosidic linkage between N-acetylglucosamine and asparagine was accomplished either chemically or enzymatically. Acid hydrolysis of the linkage moiety resulted in formation of equimolar amounts of glucosamine, ammonia, and aspartic acid. The N-glycosidic linkage was relatively stable to alkali and required more drastic conditions for cleavage than the O-glycosidic linkage (see below). The linkage was cleaved in 0.5 to 1 N NaOH in the presence of 1 to 4 N $NaBH_4$, at 80 to 100°C for usually less than 12 hours. Problems with this treatment are: 1) less than quantitative release is often obtained; and 2) further hydrolysis of carbohydrate chain beginning from the reducing N-acetylglucosaminyl end may occur. Enzymatic cleavage of the N-glycoside was accomplished by a glycosylasparaginase which was isolated in several sources (66). These enzymes were specific for the configuration and required that the asparagine possesses free amino and carboxyl groups.

Many glycoproteins containing the N-glycosidic linkage have a characteristic "core" structure of mannosyl di-N-acetylchitobiose linked to asparagine (Man-GlcNac-GlcNac-

Asn). Various glycoproteins have not been examine in sufficient detail to reveal the core structure beyond the GlcNAcAsn linkage. The oligosaccharides linked to asparagine in several glycoproteins have been sequenced completely and fall into two broad categories: the simple, which contains only mannose and N-acetylglucosamine residues; and the complex, which also contain sialic acid, galactose, and fucose. A single glycoprotein may contain both simple and complex carbohydrate chains (e.g. thyroglobulin and IgM) or it may contain both N-linked chains and O-linked chains as does fetuin (76). The core structure of GlcNAc-GlcNAc-Asn was first identified in bovine RNase (72) and since then a number of glycoproteins have been shown to have this disaccharide linked to asparagine. The newly described endoglucosaminidases, capable of cleaving this di-N-acetylchitobiose bond, were very useful in indicating the presence of this core structure. The next outermost sugar that occurs in many N-linked oligosaccharide chains of both simple and complex type is mannose. Bovine RNase B contains a simple type oligosaccharide chain $(Man)_6(GlcNAc)_2$ attached to the Asn34. Treatment with alpha-mannosidase removed all but one mannose residue leaving a $(Man)_1(GlcNAc)_2$-Asn core from which the last mannose was removed with hen oviduct beta-mannosidase (67). Similarly core mannose was removed from ovalbumin glycopeptide by Snail beta-mannosidase (68) and from

ovalbumin and Aspergillus alpha-amylase by pineapple beta-mannosidase (69). This beta-linked core mannose residue has been found in a number of other glycoproteins (55).

Although oligosaccharide chains containing only mannose and N-acetyl glucosamine are designated as "simple", deducing the sequence and linkage of some 5 to 11 alpha-mannose residues is clearly a more formidable task than deducing the sugar sequence in heterosaccharide chains of complex type. Nevertheless, structures of a group of high-mannose (simple) glycopeptides have been obtained (70). They share several common features. The inner beta-linked mannose is substituted at C-3 and C-6 by two additional mannose residues-a pattern seen in all other glycopeptides. A backbone of mannose residues linked 1-6 to each other and to beta-linked mannose was found in mannan-protein of Saccharomyces cerevisiae (71). Side chains of one to three mannose residues were attached to the backbone in alpha 1-3 linkage. The side chain mannose was linked to each other in alternating alpha 1-2, alpha 1-3 sequence. Identical patterns of core structure, branching and linkage were found in Aspergillus alpha-amylase (6 mannose residues) and ovalbumin (5 to 6 mannose residues). N-acetylglucosamine was found linked to the non-reducing end of mannose. However, exceptions to this common structure were also identified. For example, the high-mannose glycopeptide C-1 isolated from a human IgE (61) was shown to have alternating

mannose and N-acetylglucosamine residues without mannosyl-chitobiose core sturcture.

Asparagine-linked oligosaccharides of the complex type contain a variable number of outer chains linked to a core of mannose and N-acetylglucosamine. Beyond N-acetylglucosamine linked to asparagine, no common core structure was identified as was the case of N-glycosidic linkage of simple-type oligosaccharide of glycopeptides. However, Man-GlcNAc-GlcNAc-Asn sequence was identified in several glycoproteins, e.g. human IgG, IgE, and IgA_1, bovine IgG, ovomucoid, porcine thyroglobulin and bovine thymocytes, with substitutions commonly found linked to mannose or N-acetylglucosamine. A core sequence of GlcNAc--Man--GlcNAc--Asn has been suggested for human transferrin (60). Substitutions linked to the core sequence are usually fucose and N-acetylglucosamine. The sequence of outer chains was most often NeuAc-Gal-GalNAc as exemplified by various immunoglobulin glycopeptides, porcine thyroglobulin, fetuin glycopeptide, and human transferrin glycopeptides. Relevant to immunology, the human Ig glycopeptides and calf thymocytes glycopeptides have been sequenced completely. A core structure of Man-beta1-4-GlcNAc-beta1-4-GlcNAc-beta-Asn was found in human IgG, IgE, and IgA_1, and bovine IgG with substitution of fucose linked alpha 1-6 to GlcNAc. However, human IgE C-1 glycopeptide contains a core structure of GlcNAc-alpha 1-3-Man-beta1-4-GlcNAc--Asn. Branched

structure occurs at terminal mannose with alpha 1-3 and alpha 1-6 substitutions with two mannose residues. Outer chains of sequence NeuAc-alpha 1-2-Gal-beta1-4-GlcNAc-beta1-2 are linked to the non-reducing end of mannoses. The microheterogeneity occurs when outer chains are at varying stages of completion with respect to addition of galactose or sialic acid to the non-reducing ends of the oligosaccharides. Microheterogeneity may also reflect genetic differences; for example, human IgG glycopeptide B-3 contains an additional N-acetylglucosamine residue linked to branch mannose, or human IgA_1 glycopeptide IIA lacks fucose but has the extra N-acetylglucosamine on the branch mannose. Three asparagine-linked oligosaccharides of complex type were isolated from calf thymocyte delipidated membrane glycoproteins, by Kornfeld (73). The proposed structures of oligosaccharide chains contain some features common to other membrane glycoprotein oligosaccharides and serum glycoprotein oligosaccharides as well as several unique features. The branching arrangement of the mannose residues in these glycopeptides occurs in other glycoproteins such as fetuin which contains three mannose residues. Two outer chains were linked to C-3 and C-6 of an outer mannose residue. (In the case of fetuin, the two outer chains are linked to C-2 and C-4 of an outer mannose.) The outer chain sequence of NeuAc-Gal-GlcNac is commonly found whereas sequences of NeuAc-Gal-Gal-GalNAc and Gal-Gal-GlcNAc are

unique among known oligosaccharide structures.

Glycopdptides with N-acetylgalactosaminyl serine or threonine linkage.

In many glycoproteins, including mucous glycoproteins, the oligosaccharide chains are linked O-glycosidically to the hydroxyl groups of serine and/or threonine in the peptide (alpha-configuration). The sugar moiety involved in the linkage of mucous glycoproteins is N-acetylgalactosamine whereas the linkage sugar in proteoglycan is xylose (55-58). Mannose as a linkage sugar has been identified in yeast mannan (70), yeast cell wall glycoprotein (74), and fungus imperfectus envelope glycoproteins (75). In a few cases, galactose or fucose has been identified as the linkage monosaccharide residue (58). Identification of O-glycosidic linkage is based on its lability under alkaline conditions. An alkali-catalyzed beta-elimination reaction was postulated to explain the results (76). When carried out in alkaline/borohydride condition, the linkage sugar is converted to sugar alcohol and can be identified after hydrolysis of the oligosaccharide chain. The involvement of serine and threonine in O-glycosidic linkages was demonstrated by a selective loss of serine and threonine in alkali treated mucus glycoproteins. When reduced with sodium borohydride, the beta-elimination derivatives of serine and threonine are converted to alanine and alpha-aminobutyric acid. The effects of alkali on the

carbohydrate moiety of the O-glycosidic bond have been considered in detail (56). Alkali treatment of the N-acetylgalactosaminyl hydroxyamino acid linkage results in the formation of the N-acetylgalactosamine as the reducing sugar. This product is not stable in an alkaline environment and may undergo another beta-elimination reaction resulting in the formation of a furanosyl compound. If C-3 substituted, the reducing N-acetylgalactosamine is very alkali labile and successive degradation reactions will continue until alkaline-stable substance remains. This stepwise degradation is termed the "peeling-reaction". The extent of the peeling reaction can be reduced by lowering the concentration of alkali and by the addition of sodium borohydride to the reaction mixture. In the presence of borohydride the aldehydic group of N-acetylgalactosamine is converted into primary alcohol which is less sensitive to mild alkaline condition. Common conditions for beta-elimination reaction are 0.05 to 0.1 N NaOH and 1M $NaBH_4$ at 37 degree for 12 to 24 hours. These conditions usually yield intact oligosaccharide but may not produce quantitative yield of all carbohydrate chains from all glycoproteins. A second elimination reaction after isolation and reacetylation of products from the first alkali-borohydride treatment will increase the total yield of carbohydrate chains.

O-glycosidically linked oligosaccharide does not have

any constant core structure beyond the linkage sugar. The oligosaccharides are similar in composition and type of linkage to the outer chains of asparagine-linked oligosaccharide and glycolipid-bound oligosaccharides (77). The biosynthesis of O-glycosidic linkage is believed to be initiated in rough endoplasmic reticulum and elongated in the Golgi apparatus. But the mechanism is less complex and does not require dolichol intermediates (58). The initiation of O-glycosidic linkage requires a UDP-N-acetylgalactosamine: polypeptide N-acetylgalactosaminyl-transferase which has been described in ovine, porcine, and bovine submaxillary glands, HeLa cells, fibroblasts, and other tissues (58). This enzyme transfers the N-acetylgalactosamine to serine or threonine of polypeptide in O-glycosidic linkage. Glycosyltransferases such as sialyltransferase, N-acetylgalactosaminyltransferase , galactosyltransferase, and fucosyltransferase will elongate the oligosaccharide chain by adding the proper sugar moiety. Each glycosyltransferase recognizes a specific sequence of receptor oligosaccharide chains and determines the type of sequence manufactured. This type of mechanism is exemplified in the biosynthesis of Blood group antigen, A,B,H,(O) and Lewis(Le). These antigens occur as O-glycosidically linked oligosaccharides attached to mucins secreted in the gastrointestinal, genitourinary, and respiratory tracts (also as glycosphingolipids on red

blood cell membrane, or as oligosaccharides in milk and urine.) The biosynthesis of each determinant is controlled by glycosyltransferase coded for by its gene locus. Therefore the ABO locus is responsible for two transferases, the A-dependent 1,3-N-acetylgalactosaminyltransferase and the B-dependent 1,3-galactosyltransferase. Both recognize a common precursor sequence Fuc-1,2-Gal-beta-R synthesized by GDP-Fucose: beta-galactoside 1,2-fucosyltransferase coded for by H gene locus. Deficiency in fucosyltransferase will prevent the synthesis of A,B,and H determinants whereas a deficiency in either the A-dependent or the B-dependent transferase will prevent synthesis of A or B determinant.

Glycopeptides linked through the hydroxyl group of hydroxylysine.

The O-glycosidic linkage involving galactose and hydroxylysine is characteristically found in collagen and collagen-like polymers such as Clq component of the complement system. Butler and Cunningham (78) first demonstrated the presence of the galactosylhydroxylysine linkage in soluble collagen from guinea pig skin. An equal molar amount of glucose was also found in the glycopeptide as galactosylhydroxylysine linkage. Structural studies of the disaccharide prepared by mild acid hydrolysis following labilization of the glycosidic bond attached at the -OH group by N-acetylation of amino group of hydroxylysine, have shown the structure of D-glucopyranosyl-1,2-beta-D-galactopyranosylhydroxylysine. The

disaccharide-hydroxylysine-containing glycopeptide of human glomerular basement membranes has been shown to carry major antigenic determinant for autosensitization in some case of glomerulonephritis.

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NEURAMINIDASE SENSITIVITY OF THY-1 ACTIVE GLYCOCONJUGATES

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Abstract—Three forms of Thy-1 active antigens were studied for their relative sensitivity to the enzyme neuraminidase (Nase). Those studied were Thy-1 active shed material, Thy-1 active glycolipids and Thy-1 active glycoprotein. Each expressed either Thy-1.1 or Thy-1.2 allotype according to the mouse strain of origin. Thy-1 activity was assessed by a plaque forming cell (PFC) assay specific for Thy-1 allotypes and by a solid phase non-competitive immunoradiometric assay (IRA) specific for Thy-1.2. Treatment of Thy-1 active complexes shed from either C3H (Thy-1.2) or AKR (Thy-1.1) thymocytes with Nase resulted in the loss of respective Thy-1 antigenicity in both PFC assay and the direct binding IRA. The antigenicity decreased significantly within the first few hours and the loss of Thy-1 activity was complete by 24 hr. Thy-1.1 and 1.2 activity of glycolipids was also destroyed by Nase treatment, and the Thy-1.2 activity of purified Thy-1.2 glycoprotein was also destroyed by Nase treatment as assessed by the PFC assay. An unusual finding was that both Thy-1.1 active shed complexes and Thy-1.1 glycolipid after Nase treatment exhibited Thy-1.2 activity. This conversion was confirmed using two forms of Thy-1.1 for the PFC assay (which relies on the immune response to Thy-1) and for the IRA (which depends only on direct binding of anti-Thy-1.2 to the antigen). The sensitivity of all three forms of antigen was the same regardless of the source of enzyme and three sources of Nase enzymes catalysed the conversion of Thy-1.1 into Thy-1.2 active forms. These results suggest that sialic acid may be necessary but not sufficient for the expression of Thy-1 antigenicity.

INTRODUCTION

Thy-1 antigen was first identified by Reif & Allen (1966) as a differentiation antigen on mouse thymocytes and brain cells. Two allotypes of Thy-1 antigen have been identified, i.e. Thy-1.1 antigen in RF and AKR mice and Thy-1.2 antigen in C3H and most other strains of mice. No serological cross-reactivity was observed between these two types when murine anti-Thy-1 alloantisera were used. However, some common non-Thy-1 antigenic determinants have been identified with xenogenic 'anti-Thy-1' antisera (Williams *et al.*, 1977). Cells with Thy-1 antigenicity were also found in other species of mammals such as the rat (Williams *et al.*, 1977) and at low levels in mouse epidermal cells and fibroblasts (Scheid *et al.*, 1972; Stern, 1973).

The chemistry of murine Thy-1 has been investigated and two species of molecules have been isolated and purified which carry Thy-1 antigenicity—glycoproteins and glycolipids. Thy-1.1 active glycoprotein has been prepared from rat thymocytes and brain and has been extensively characterized (Williams *et al.*, 1977). This molecule is glycoprotein in nature with a molecular weight of 18,000 daltons and a 30% carbohydrate composition. The rat Thy-1.1 polypeptide has been sequenced and appears to have some sequence homology with heavy chain constant region of immunoglobulin (Campbell *et al.*, 1979). Similar glycoproteins have been isolated from lymphoma cell lines and from mouse brain and thymocytes (Zwerner *et al.*, 1977; Letarte & Meghji, 1978). The antigenic determinants of Thy-1 have been reported to be protein (Williams *et al.*, 1977) or carbohydrate in nature (Trowbridge & Hyman, 1975).

The antigenicity of unpurified and purified Thy-1.1 active glycoprotein was destroyed by extensive protease treatment (Williams *et al.*, 1977), although this may have been due to the reduced activity of glycopeptides compared to intact protein. Carbohydrates have also been suggested to be factors in the Thy-1 determinant (Vitetta *et al.*, 1974; Trowbridge & Hyman, 1975). Johnson *et al.* (1976) reported the neuraminidase sensitivity of crude Thy-1 preparations from lymphoblastoid cells. We have isolated and partially characterized glycolipids from murine lymphoma cell lines, brain and thymocytes which express the Thy-1.1 or 1.2 allotypes (depending on the strain of origin) (Wang *et al.*, 1978; Kato *et al.*, 1979). These

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glycolipids have the properties of gangliosides, in that they contain sialic acid, and we have proposed that the Thy-1 antigenic determinants are found in carbohydrate which may be bound to on either protein or lipid carriers. It has previously been demonstrated that the plaque forming cell (PFC)* assay specific for Thy-1 can detect very small amounts of Thy-1 in the glycolipid or glycoprotein forms (Wang *et al.*, 1978) as well as in membrane complexes shed from lymphoma cells or thymocytes (Freimuth *et al.*, 1978). We have used this technique to characterize the Thy-1 active glycolipids of both brain and lymphoma cells with regard to their thin layer mobility and their sialic acid content (Kato *et al.*, 1979). We now report the effect of neuraminidase treatment on three forms of Thy-1 antigen as assessed by the Thy-1 immune response–PFC assay and by a direct immunoradiometric assay.

MATERIALS AND METHODS

Preparations of Thy-1 active glycolipid, glycoprotein and shed material

C3H/HeJ and AKR/J mouse brain Thy-1 glycolipids (gl) were prepared following the procedure of Wang *et al.* (1978). Mouse brains were homogenized and extracted with chloroform–methanol mixtures followed by partition of the extracts into two phases (Esselman *et al.*, 1972). The ganglioside-rich upper phase was collected, lyophilized and then separated by Anasil S column chromatography (Laine *et al.*, 1977). G_{M1} ganglioside rich fractions (containing Thy-1 gl) were collected and further purified by thin layer chromatography (Silica gel 60 using Solvent 1: chloroform–methanol–2.5 *N* NH_4OH, 60:35:8). Thy-1 glycolipid migrated with G_{M1} in this system (Kato *et al.*, 1979). G_{M1} and Thy-1 were then separated by thin layer chromatography on Silica gel 60 in Solvent 2 (Chloroform–methanol–0.02% $CaCl_2$ 55:40:9). Thy-1 gl prepared in this way was contaminated with G_{D1a} and this was removed by additional t.l.c. fractionation in Solvent 2. In some experiments two dimensional t.l.c. purified Thy-1 glycolipids were used for Nase treatment (Kato *et al.*, 1979).

Thy-1.2 active glycoprotein has been prepared from C57BL 6 brain. Details of purification have been reported elsewhere (Letarte & Meghji, 1978). In brief, mouse brains were solubilized with Triton X-100. The extract was subjected to *Lens culinaris* lectin affinity chromatography. The bound glycoprotein was then eluted with methyl-α-D-mannoside in deoxycholate buffer. Fractions containing Thy-1 activity were pooled, concentrated and rerun on AcA 34 column. Glycoproteins were precipitated with 70% (w/w) ethanol, concentrated and used for experiments in phosphate buffered saline.

The preparation of Thy-1 active shed material was similar to that described by Lake (1976). Cells obtained from either C3H or AKR mouse thymuses were cultured at 10^7 cells per ml serum free Dulbecco's modified minimal essential medium (D-MEM) at 37°C, 8% CO_2, humid incubation for 1.5–2 hr (cell viability was near 98%). After incubation, the shed material was collected by centrifuging the cells at 300 *g*. Supernatants were gathered and kept frozen at −70°C until the time of use.

Neuraminidase treatment

Clostridium perfringens neuraminidase (CpNase) was obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.). and *Vibrio cholera* neuraminidase (VcNase) was obtained from Koch-Light Laboratories (Bucks, U.K.) and *Arthrobacter ureafaciens* neuraminidase (AuNase) was obtained from EY Laboratories (San Mateo, CA, U.S.A.). The enzymes were used without further purification. The Thy-1 glycolipid obtained from 10 g of mouse brain was incubated with 0.15 units of Cp Nase (or 25 units of VcNase) in 200 μl of acetate buffer, pH 4.5. The mixture was incubated at 37°C for various periods of time. At the end of incubation, the enzyme reactions were stopped in a boiling water bath for 1 min. The samples were dried and extracted with chloroform–methanol (2:1). Aliquots of the neuraminidase-treated Thy-1 glycolipids were then tested for Thy-1 antigenicity in the anti-Thy-1 plaque-forming cell assay.

Thy-1 active glycoprotein (0.1 μg) was incubated with 0.15 units of CpNase or 25 units of VcNase under the same conditions as described above. After incubation, the enzyme reaction was stopped in a boiling water bath for 1 min. Samples were assayed

* Abbreviations: PFC, plaque forming cell; IRA, immunoradiometric assay; Nase, neuraminidase; Cp, *Clostridium perfringens*; Vc, *Vibrio cholera*; Au, *Arthrobacter ureafaciens*; FCS, fetal calf serum; BSA, bovine serum albumin; G_{M1}, GalGalNAGal(NANA)Glc Ceramide.

without further treatment for anti-Thy-1 PFC response.

VcNase was immobilized on Sepharose 4B beads (Pharmacia) before use with C3H or AKR thymocyte shed material in the PFC assay. Sepharose 4B beads were first activated by cyanogen bromide (March *et al.*, 1974) and VcNase (250 units) was bound to 2 ml of activated beads. The beads were assayed for Nase activity by incubating with [1-^{3}H]neuraminlactitol (New England Nuclear). The conversion of neuraminilactitol to lactitol was measured by t.l.c. The shed materials were then incubated with VcNase-Sepharose 4B beads for various times, centrifuged and the supernatant was collected and assayed. Each sample was divided into two equal parts and assayed using the PFC assay or the direct IRA. Shed material was also treated with Nase and assayed directly by IRA as described below.

Anti-Thy-1 plaque-forming cell assay

The PFC assay for Thy-1—first developed by Fuji et al. (1970). We have previously used this assay to study mouse brain and thymus Thy-1 active glycolipid preparations and the details of the assay have been stated elsewhere (Wang *et al.*, 1978). Both primed and unprimed mice were used in these experiments. AKR/J or C3H/HeJ mice to be primed were immunized with 4×10^7 thymocytes from the strain of the opposite allotype. Two to three weeks after immunization these mice were killed and their spleens were used for the anti-Thy-1 PFC assay. Splenocytes (primed or unprimed) were cultured with Thy-1 glycoconjugates in Marbrook culture vessels with 9 ml CMRL/FCS media in the outer chamber. Marbrook vessels were cultured in a CO_2 incubator (9%) at 37°C for 4 days before assaying for anti-Thy-1 PFC response. Cells from Marbrook vessels were plated in a lawn of 2×10^7 thymocytes of appropriate Thy-1 type in 0.6% agarose solution. Anti-Thy-1 antibodies from antibody-producing B cells produced plaques after the addition of 1:10 diluted rabbit complement. The plaques were visualized by trypan blue exclusion and counted under a dissection microscope.

Immunoradiometric assay of Thy-1 antigen

A direct binding solid phase immunoradiometric (IRA) used for detecting Thy-1 antigens has been described in detail elsewhere (Schwartz *et al.*, 1980). Briefly, the procedure involves coating the walls of microtiter wells with antigen, followed by binding of ^{125}I-labelled antisera. A monoclonal anti-Thy-1.2 antibody with a titer of about 10^{-6} was obtained from New England Nuclear (Boston, MA, U.S.A.) and used in these experiments. This antiserum was found to be highly specific for Thy-1.2 negative lymphoma cells (S49.1) and normal thymocytes of CBA mouse but not for Thy-1.2 negative cells or Thy-1.1 cells. The monoclonal antibody was iodinated with carrier-free NaI by the Chromine-T method (Greenwood *et al.*, 1963). After iodination, unreacted iodine was removed by Sephadex G-25 chromatographic separation. Toluene etched microtiter plates (Dynatech Laboratory, Inc., Alexandria, VA. U.S.A.) were coated with Thy-1 active thymocyte culture supernatants by allowing 100 μl of thymocyte supernatants to stand in each well for 10 hr. Each well was then washed twice with phosphate buffered saline (PBS) and incubated with 10% bovine serum albumin (BSA) for 10 min. Then the wells were washed twice, and 100 μl of ^{125}I-labelled antibody (in 5% BSA solution) was added to each well and incubated for one hr. Approximately one million counts were added to each well. After incubation, each well was washed six times with PBS, sliced free from the tray, and then counted with a gamma counter. Neuraminidase was added to culture supernatant coated wells. After various times of incubation, each well was washed twice and tested for remaining Thy-1.2 antigenicity with ^{125}I-labelled antiserum.

RESULTS

Thy-1 active shed complexes

Supernatant media from Thy-1 positive thymocytes and lymphoma cells have been used as a source of Thy-1 active membrane material and the PFC response to this material has been shown to be readily induced and specific for Thy-1 alloantigen (Freimuth *et al.*, 1978; Lake, 1976). We treated both Thy-1.1 and Thy-1.2 shed material with VcNase linked to Sepharose 4B beads to determine the susceptibility of Thy-1 antigenic determinants to neuraminidase. The VcNase treatment decreased Thy-1 antigenicity of Thy-1.1 shed material from 128 ± 6 PFC to 5 ± 3 PFC in the anti-Thy-1.1

Table 1. Neuraminidase treatment of Thy-1 active antigen

Antigen	Treatment[a] *Vibrio cholera Nase*	PFC/10^7 cells[b] anti-Thy-1.1	anti-Thy-1.2
Shed membrane			
Thy-1.1 Shed	None	128 ± 67	0 ± 0
	+	5 ± 3	136 ± 23
Thy-1.2 Shed	None	2 ± 1	159 ± 38
	+	6 ± 5	9 ± 5
Glycolipid			
Thy-1.1 gl	None	41 ± 13	2 ± 1
	+	11 ± 11	79 ± 10
Thy-1.2 gl	None	0 ± 0	75 ± 14
	+	0 ± 0	15 ± 9
Glycoprotein			
Thy-1.2 gp	None	10 ± 11	82 ± 34
	+	—	19 ± 9

[a]Incubation was done at 37°C for 24 hr.
[b]Values shown are the means ± S.E. of five cultures.

response (Table 1). Similar results were also obtained with VcNase-treated Thy-1.2 shed material (9 ± 5 PFC after treatment in comparison to 158 ± 38 PFC induced with intact Thy-1.2 shed material). These results, with the controls shown in Table 1, demonstrate the specificity of the PFC response. When Thy-1.1 shed material was treated with Nase, however, the resulting material had Thy-1.2 activity and no Thy-1.1 activity. The Nase converted Thy-1.1 shed material induced 136 ± 23 PFC in the anti-Thy-1.2 response. There was no Thy-1.1 antigenicity found in Nase-treated Thy-1.2 shed material (6 ± 5 PFC).

This apparent conversion was further studied by an analysis of the kinetics of disappearance and appearance of Thy-1 activity. Thy-1 shed materials were incubated with VcNase coupled Sepharose 4B beads and after various periods of time the supernatants were collected by centrifugation. The samples were assayed in both the anti-Thy-1.1 and anti-Thy-1.2 PFC assays (Figs. 1 and 2). Thy-1.1 active shed material lost more than 80% of antigenicity within the first 3 hr of incubation and after 24 hr no significant activity remained. When the same samples were assayed in Thy-1.2 response, the Thy-1.2 antigenicity increased slowly during the first 12 hr and then more rapidly until the level was indistinguishable from the response of normal Thy-1.2 shed material. The Thy-1.2 shed material also lost its antigenicity within the first 6 hr of Nase incubation, decreasing from 179 ± 38 PFC to 47 ± 9 PFC. After 24 hr there was no significant antigenicity. No Thy-1.1 antigenicity was found in Nase-treated Thy-1.2 shed material.

Because of the complexity and subjectivity of the PFC response we developed a direct binding immunoradiometric assay to study Thy-1 antigen conversions. For these experiments Thy-1.1 and 1.2 shed material was bound di-

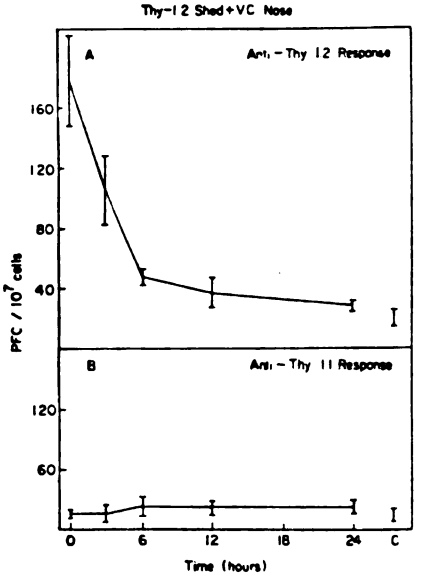

Fig 1. Thy-1.2 active shed complexes treated with *Vibrio cholera* neuraminidase. Shed material collected from 10^8 C3H thymocytes was treated with VcNase bound to Sepharose 4B beads. Fractions were collected at different intervals. After removing the beads by centrifugation, each fraction was divided equally into anti-Thy-1.2 (A) and anti-Thy-1.1 (B) PFC assays. Controls (C) consisted of cultures containing no antigens. Data presented here are the means ± S.E. of five cultures.

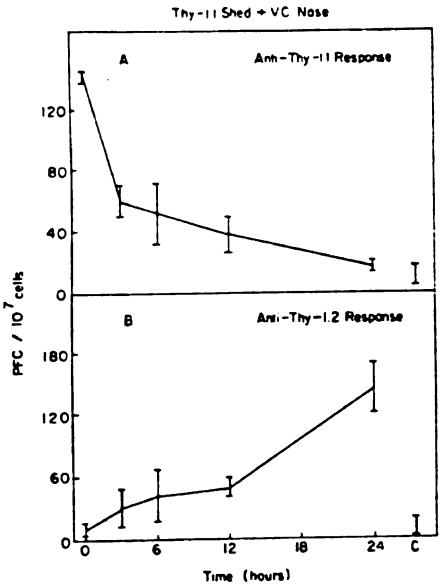

Fig 2. Thy-1.1 active shed complexes treated with *Vibrio cholera* neuraminidase. The VcNase treatment of Thy-1.1 active material was identical to that in Fig 1. Each fraction of VcNase treated Thy-1.1 active shed material was divided equally into both anti-Thy-1.1 (A) and anti-Thy-1.2 (B) PFC assays. Labels are identical to those in Fig. 1. Data shown are the means ± S.E. of five cultures.

rectly to plastic microtiter plates. Nase was added and incubated for various times in the wells, followed by washing and addition of iodinated anti-Thy-1.2 serum. The binding of anti-Thy-1.2 to Nase-treated Thy-1.2 is shown in Fig. 3(A). VcNase completely destroyed Thy-1.2 activity within 24 hr with kinetics which appeared somewhat slower than with the PFC assay. Thy-1.1 antigen before treatment with Nase had no Thy-1.2 activity but after this treatment Thy-1.2 activity increased up to 50 hr (Fig. 3B). Similar sensitivity and interconversion of Thy-1 with Nase treatment was observed using *Clostridium perfringens* Nase and affinity purified *Arthrobacter ureafaciens* Nase. The conversion of Thy-1.1 active material into Thy-1.2 active material has been demonstrated using two independent assays and three sources of enzyme.

Neuraminidase treatment of Thy-1 active glycoconjugates

Thy-1.1 and Thy-1.2 active glycolipids have previously been reported and partially characterized using the Thy-1 specific PFC assay (Wang *et al.*, 1978; Kato *et al.*, 1979). Treatment of Thy-1.1 or Thy-1.2 glycolipid with VcNase resulted in a loss of antigenicity within 24 hr (75 ± 14 to 15 ± 10 PFC for Thy-1.2 and 41 ± 14 to 11 ± 12 PFC for Thy-1.1) (Table 1). When Nase-treated Thy-1.1 was tested for Thy-1.2 activity a good response of 79 ± 11 PFC (compared to 2 ± 1 PFC for intact Thy-1.1) was observed. On the other hand, Nase-treated Thy-1.2 did not exhibit any Thy-1.1 activity.

Thy-1.1 and Thy-1.2 glycolipids were treated with Nase at different intervals of time and assayed for remaining specific Thy-1 antigenicity. Thy-1 antigenicity decreased rapidly over the first 10 hr of treatment (Fig. 5A) and then decreased slightly to insignificant levels between 10 and 48 hr. Similar kinetic patterns were also obtained with Thy-1.2 glycolipid as shown in Figs. 4(A) and 6(A). Thy-1.1 and Thy-1.2 glycolipids were also assayed after

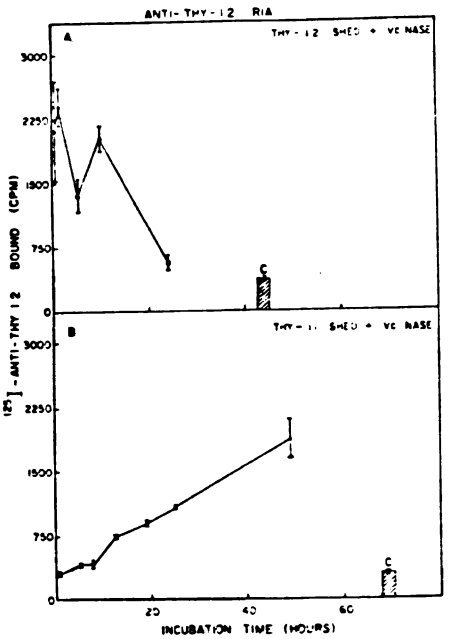

Fig. 3. Direct binding IRA assay for VcNase treated Thy-1.1 and Thy-1.2 active shed complexes. Shed complexes were bound to microtiter plates and treated with VcNase as described in Materials and Methods. Both Thy-1.2 (A) and Thy-1.1 (B) Nase treated complexes were assayed for Thy-1.2 activity. Each point represents the mean ± S.E. of triplicate samples.

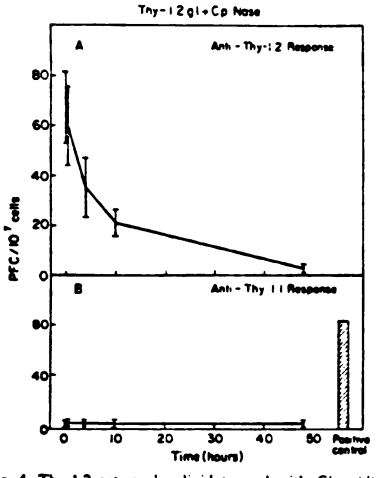

Fig. 4. Thy-1.2 active glycolipid treated with *Clostridium perfringens* neurneuraminidase. Thy-1.2 active glycolipid prepared from a two-dimensional t.l.c. system was used. CpNase (0.5 units) in 500 μl of acetate buffer (pH 4.5) was added to a glass tube containing dried Thy-1.2 glycolipid prepared from C3H mouse brain. At different intervals, samples were collected and heat-inactivated. Each sample was equally divided and assayed in anti-Thy-1.2 PFC response (A) and anti-Thy-1.1 PFC response (B). Data shown are the means ± S.E. of five cultures.

Nase treatment for anti-Thy-1.2 PFC and anti-Thy-1.1 PFC response, respectively. Nase treatments of Thy-1.1 glycolipid gradually induced the increase of Thy-1.2 antigenicity (Figs. 5B and 6B) whereas Nase treatment of Thy-1.2 did not show any Thy-1.1 activity (Fig. 4B). This confirms the observation that Nase converted Thy-1.1 antigen into a compound carrying Thy-1.2 antigenicity using highly purified glycolipid rather than complex shed material as discussed above.

Results of Nase treatment of Thy-1 was also confirmed using a highly purified Thy-1.2 active glycoprotein isolated by lentil lectin chromatography from mouse brain. Treatment of this antigen with Nase resulted in the decrease of antigenic activity from 82 ± 34 to 19 ± 9 PFC within 24 hr (Table 1). The susceptibility of Thy-1.1 glycoprotein was not assessed because it was not available in purified form.

DISCUSSION

The immunochemical properties of Thy-1 antigen were studied using three forms of the antigen which include complexes, shed material, glycolipid and glycoprotein. The use of shed material was of interest because it could be obtained easily and because other membrane determinants such as H2 were found in association with it. The Thy-1 specific immune response to shed material from CBA and AKR thymocyte culture medium was first studied by Lake (1976). Shed material was found to induce specific anti-Thy-1 PFC according to its original alloantigen type. High PFC numbers were reported, 200–400 PFC's per culture in comparison to 60 – 150 PFC's that we observed with purified glycoproteins and glycolipids. The anti-Thy-1 PFC assay with complex antigen required specific conditions for a high response. These include identical H-2 haplotype (at least K and D alleles) between donor and recipient, background differences in some non-H-2 cell surface antigens and T cell help (Lake & Douglas, 1978; Zaleski & Gorzynski, 1978).

We have studied the effect of neuraminidase treatment on shed complexes, and both Thy-1.1 and Thy-1.2 shed material lost their antigenicity within 24 hr of incubation. Nase-treated Thy-1.1 shed complexes lost Thy-1.1

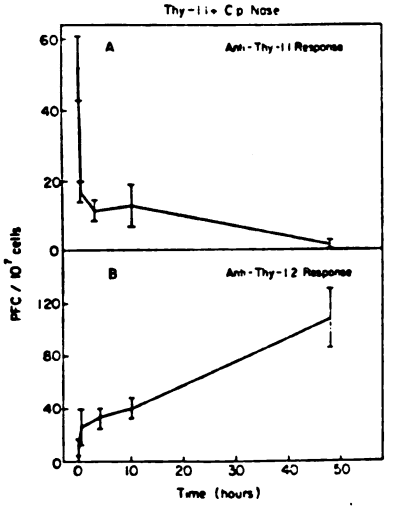

Fig. 5. Thy-1.1 active glycolipid treated with *Clostridium perfringens* neuraminidase. Two dimensional t.l.c. purified Thy-1.1 glycolipid was treated with Nase as described in Fig. 4. At different intervals, fractions were collected and divided equally into anti-Thy-1.1 PFC assay (A) and anti-Thy-1.1 PFC assay (B). Data shown are the means ± S.E. of five cultures.

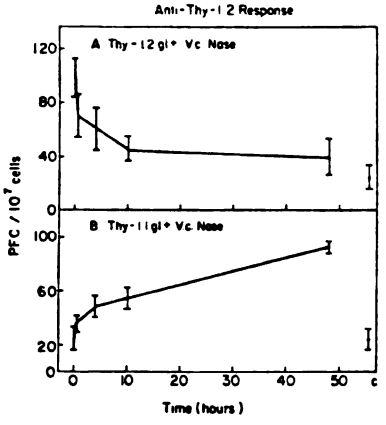

Fig. 6. Thy-1.2 and Thy-1.1 active glycolipids treated with *Vibrio cholera* neuraminidase. Thy-1.2 or Thy-1.1 active glycolipid was incubated with 50 units of VcNase in 500 μl acetate buffer (pH 4.5) and treated as described in Fig. 4. Thy-1.2 glycolipid (A) and Nase treated Thy-1.1 glycolipid (B) fractions were assayed in the anti-Thy-1.2 PFC assay. Controls (C) consisted of cultures containing no antigens. Data shown are the means ± S.E. of five cultures.

determinant and converted to or became cross-reactive with Thy-1.2. The loss of Thy-1 PFC response of the shed material could be due to other cell surface antigens affected by Nase which fail to facilitate the recognition of responder cells for the opposite Thy-1 type. This possibility was eliminated, however, by corroboration of the *in vitro* PFC results with the direct binding anti-Thy-1.2 IRA which does not depend on the immune response to Thy-1. The results of the IRA were essentially identical to those obtained with the PFC assay except for slight variation in the rate of decrease of anti-Thy-1.2 binding (Fig. 3). Anti-Thy-1.2 binding to C3H thymocyte shed material was completely removed by Nase treatment and anti-Thy-1.2 binding capacity appeared after Nase treatment of AKR thymocyte shed material. Thus, by two totally independent means, Thy-1.2 antigen normally found in the membrane was Nase labile and Thy-1.1 antigen could be converted into Thy-1.2 active material by Nase treatment. The naturally occuring Thy-1.2 molecule has different properties from the Thy-1.2 derived by Thy-1.1 because the latter was partially resistant to Nase. This suggests that although sialic acid may be intimately involved in the expression of the Thy-1.2 active determinant it may not be essential to Thy-1.2 activity.

Purified Thy-1 active glycolipids were studied in conjunction with Thy-1 active shed material to determine if the results obtained with complex antigen (consisting of both Thy-1 active glycolipids and glycoproteins as well as many membrane components) could be duplicated with pure antigen. Thy-1.1 and Thy-1.2 glycolipids were both found to be similar to shed material in that they were labile to Nase treatment within 24 hr. The kinetics of Nase action demonstrated a rapid decrease of respective antigenicity in the first 4 hr and no significant antigenic activity was observed after 48 hr of treatment. This suggests that both Thy-1.1 glycolipid and Thy-1.2 glycolipid contain neuraminic acid moieties and that the removal of these moieties alters their antigenicity. Neuraminidase treatment of Thy-1.1 purified glycolipid also caused the appearance of Thy-1.2 antigenicity which previously had been masked. The enzyme induced development of the Thy-1.2 antigenicity was apparently slower than the destruction of the original Thy-1.1 antigenicity, and the Thy-1.2 antigenicity increased in a linear fashion between 10 min and 48 hr of Nase treatments (Figs. 2B and 5B). This could be because anti-Thy-1 PFC assay responded to a moderate range of antigen and the dose we used was just enough to give maximum PFC response. The conversion of a small percentage of Thy-1.1 glycolipid would decrease the anti-Thy-1.1 response drastically, while the amount converted would not be enough to induce significant anti-Thy-1.2 response. Only after sufficient converted Thy-1.1 glycolipid had accumulated through longer Nase treatment would it induce significant anti-Thy-1.2 PFC response.

The results obtained with shed material and purified glycolipid were further confirmed using purified Thy-1.2 active glycoprotein. The Thy-1.2 activity of the glycoprotein was quickly destroyed by treatment with Nase from either source. Sufficiently pure Thy-1.1 glycoprotein was not available to test for conversion to Thy-1.2 activity but Thy-1.1 glycoprotein in impure form exhibited the same properties as both the pure Thy-1.1 glycolipid and Thy-1.1 shed material (which contains Thy-1.1 glycoprotein).

Previously we have shown that the Thy-1 active glycolipids have the properties of gangliosides (i.e. containing sialic acid) (Wang *et al.*,

1978; Kato *et al.*, 1979). They were found to be glycolipid in nature, were isolated with other gangliosides in Folch upper phase, and had acidic properties as determined by DEAE-Sephadex ion exchange chromatography. The Thy-1 active glycolipids incorporated radioactive precursors for sialic acid ([^{14}C]*N*-acetylmannosamine and [^{14}C]glucosamine) and the radioactive label in the active compounds was found in sialic acid. These results suggested that sialic acid may be involved in the expression of Thy-1 antigen and led us to the use of neuraminidase to study Thy-1.

Neuraminidase has been used by a number of investigators to study the expression of Thy-1 on whole cells. Schlesinger & Gottesfeld (1971) originally found that the expression of Thy-1 on thymocytes was unchanged by Nase treatment while the expression of H-2 was increased. Johnson *et al.* (1976) reported that Thy-1 activity of papain released antigen could be destroyed by Nase treatment. Milewicz *et al.* (1976), on the other hand, found that the expression of Thy-1 could be induced on an intact purified population of bone marrow cells which did not previously express Thy-1. The discrepancy between these findings is undoubtedly due to the problems of using Nase with intact cells (reviewed in Milewicz *et al.*, 1976) and to the differences of using Nase between intact cells and more pure antigens. Thy-1 could be induced to appear on an intact cell by removal of accessible sialic acid on the surface of the membrane which is not involved in Thy-1. Many antigens (especially glycolipid antigens) may be hidden or cryptic in the membrane and not be readily accessible to enzyme action (Gahmberg & Hakomori, 1973). This may be especially true if there is a large amount of surface sialic acid which could compete for the available Nase. In the present report these problems were circumvented by use of purified antigens and direct assays for Thy-1 activity.

The loss of Thy-1 antigenicity after Nase treatment does not necessarily mean that neuraminic acid moieties were part of the antigenic determinants. Removal of neuraminic acid decreases the charge of the molecule and also may convert the glycolipids into water-soluble forms which might not be detected in the Thy-1 PFC assay. In addition, commercially available CpNase could be contaminated with other enzymatic activities (such as protease or endo-B-*N*-acetylglucosaminidase and protease; Chien *et al.*, 1975). To avoid problems of this nature VcNase treatment was performed in parallel to CpNase treatment. Also affinity purified *Arthrobacter ureafaciens* Nase, which has been shown to be free of glycosidases and proteases, was used and gave essentially identical results to other sources of Nase.

In summary, we have found that Nase treatment of Thy-1.1 unmasked determinants which express Thy-1.2 activity in the PFC and IRA assays. We have compared the purified Thy-1 glycolipids, Thy-1 glycoprotein and shed complexes for respective Thy-1 antigenicity and all have similar antigenicities and similar sensitivity to Nase. This similarity in susceptibility to Nase treatment supports the notion that all three forms of Thy-1 antigen carry similar if not identical antigenic determinants.

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IDENTIFICATION OF TWO DISTINCT THYMOCYTE GLYCOPROTEINS BEARING THY-1.2 DETERMINANTS[1]

By

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[1] Supported by a Grant from the National Institutes of Health, National Cancer Institute (CA 24437).

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[3] Abbreviations PBS, Phosphate buffered saline; SDS-gel, sodium dodecylsulfate polyacrylamide gel; NEPHGE, nonequilibrium pH-gel electrophoresis; PFC, plaque forming cell; Nase, Vibrio cholera neuraminidase.

ABSTRACT

The heterogeneity and antigeneic expression of Thy-1.2 glycoproteins were studied using two different monoclonal antibodies specific for the Thy-1.2 determinants. The xenogeneic anti-Thy-1.2 (clone 30-H12) and allogeneic anti-Thy-1.2 (clone HO-13.4-2.2) antibodies precipitated glycoproteins of indistinguishable relative molecular mass from an NP-40 lysate of iodinated thymocytes. After neuraminidase (Nase) treatment the quantity of alloantibody precipitate was partially reduced but the xenoantibody precipitate was unchanged suggesting a sialic acid related difference in recognition of Thy-1.2 molecules. The Mr of the precipitate was unchanged after Nase treatment. Nonequilibrium pH gradient electrophoretic analysis (NEPHGE) of the NP-40 lysate of whole cells exhibited charge heterogeneity of Thy-1.2 (at least 5 components) which decreased to two different components after Nase treatment. Both of these Nase products were precipitable with the xenogeneic anti-Thy-1.2 but only the most basic component was precipitable with allogeneic anti-Thy-1.2. The presence of two distinct Thy-1.2 bearing glycoproteins was also shown by NEPHGE analysis of immunoprecipitates of the NP-40 lysate. These antibodies both precipitated a basic family of components with charge heterogeneity but the precipitates differed in their overall pI with the allogeneic antibody precipitating the most basic components. The recognition of

different glycoproteins by these two antibodies was further verified by treating the respective immunoprecipitates with mild acid to chemically remove sialic acid. The asialo form of the glycoprotein precipitated by the alloantibody was more basic than that of the xenogeneic precipitation. Thus we have concluded that the allogeneic anti-Thy-1.2 precipitates only one of the two forms of Thy-1.2 glycoprotein of the thymocyte membrane and this more basic form is present in very small amounts compared to the total glycoprotein recognized by the xenoantibody. The xenogeneic anti-Thy-1.2 recognizes the major Thy-1.2 active glycoprotein present as well as the minor componenet. The presence of two distinct glycoproteins with Thy-1.2 activity suggests that a reevaluation of monoclonal anti-Thy-1 antibodies may reveal heterogeneity of T cell membrane glycoproteins not previously recognized.

INTRODUCTION

Thy-1 antigen was first described by Reif and Allen (1) in the thymus and brain of C3H and AKR mice. The tissue distribution in the immune system of the mouse is generally restricted to thymocytes and thymus-derived T lymphocytes and is related to the maturation and differentiation of functional T lymphocytes. Antigens similar to Thy-1 (or cross reactive with Thy-1) have been identified in lymphoid, brain and epidermal tissue of the rat, dog, and human (2-6)

but the cellular distribution in these species, especially in lymphocytes appears different from that of mouse. Thy-1 antigenic determinants have been proposed to exist on two types of glycoconjugates. Glycolipids with Thy-1 activity have been isolated from mouse brain, thymocytes and lymphoma cells but these compounds have not been fully characterized (7,8). Thy-1 active glycoproteins have been isolated from brain, nature of the Thy-1 antigenic determinants (ie. carbohydrate or protein) is as yet unclear.

Mouse lymphocyte Thy-1 glycoprotein (also designated T25) has an apparent Mr of 25 to 30,000 Da and has been found to be heterogeneous with respect to isoelectric point. Using a two-dimensional gel electrophoresis system Ledbetter et al. (13) demonstrated that Thy-1 glycoproteins from mouse thymocytes were resolved into a family of closely related glycoproteins of 25,000 to 30,000 Da with extensive charge and size heterogeneity. Neuraminidase (Nase)[3] treatment of the thymocytes followed by pH anlysis by gradient electrophoresis indicated a decrease in the heterogenity of Thy-1 glycoproteins, suggesting that the observed charge variations were caused by sialic acid. Hoessli et al. (14,15) reached a similar conclusion by using the same gel electro-phoresis system and also observed different charge heterogeneity of Thy-1 between thymocytes and lymph node cells.

We have previously reported that neuraminidase

treatment of purified Thy-1 glycoprotein or Thy-1 active glycolipid reduced Thy-1 antigen expression (16). This was assesed by first treating the antigens with neuraminidase followed by measurement of the remaining Thy-1 by the induction of a Thy-1 specific allogeneic immune reponse measured by a plaque forming cell assay (17). The results suggested that sialic acid residues were important for the expression or the presentation of Thy-1 determinants. Similar results were obtained using radioiodinated monoclonal anti-Thy-1.2 antibody in a direct binding radioimmunoassay. Experiments described in this report were designed to further examine the sensitivity of Thy-1 glycoprotein antigenic determinants to Nase treatment and to study the role of sialic acid in the observed heterogeneity of Thy-1 glycoproteins.

MATERIALS AND METHODS

Cells and preparation of radioiodinated cell surface glycoproteins. Thymocytes were removed from C3H/HeJ and AKR/J (Jackson Laboratories, Bar Harbor, ME) strains of mice and single cell suspensions were prepared by passage through a 25 guage needle. Radiolabeling of cell surface glycoproteins and proteins was performed as described previously (18) with minor modifications. Briefly, 7 X 10^7 thymocytes were incubated with 120ul lactoperoxidase (1mg/ml in PBS, Sigma, St. Louis, MO) and 500uCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston, MA 15mCi/ug) was added. The reaction was initiated by the addition of 15ul of 10mM H_2O_2. The cell suspension was mixed and incubated at 32°C for 5 minutes. The cells were then washed with PBS containing 5mM KI. Lysis of cells was performed with addition of 1% NP-40 in PBS to solubilize membrane glycoproteins. Insoluble material was removed by centrifugation at 10,000xg for 30 minutes.

Neuraminidase Treatment. NP-40 solubilized radiolabeled glycoproteins were treated with indicated amounts of Vibrio cholera neuraminidase (Calbiochem., La Jolla, CA) for various period of time. At the end of the incubation, the enzyme reaction was terminated by heating in 100°C water bath for 10 minutes. The solution was

cleared by centrifugation at 150,000xg for 30 minutes. NP-40 solubi-lized glycoproteins remained in the supernatant through this proceduore The glycoproteins were recovered by precipitation with 75% ethanol at minus 20°C and centrifugation at 150,000xg for 60 minutes. The precipitate was redissolved in 1% NP-40 in PBS and tested by immunoprecipitation. For SDS-gel electrophoresis or NEPHGE experiments, the precipitate was solubilized in respective sample buffer. Control experiments were performed simultaneously after the addition of heat inactivated Nase followed by the steps described above.

Immunoprecipitation and radioimmunoassay. Two allogeneic monoclonal antibodies used in these studies were anti-Thy-1.2 (clone HO-13.4-2.2) and anti-Thy-1.1 (clone HO-22.1.6) (19) both obtained from New England Nuclear, (Boston, MA). The xenogeneic antibody used was an anti-Thy-1.2 (clone 30-H12) (13) obtained from Becton Dickinson, Sunnyvale, CA. The xenogeneic anti-Thy-1.2 used in some experiments was isolated from clone 30-H12 obtained from the Salk Cell Distribution Center, La Jolla, CA. Radiolabeled, NP-40 solubilized cell surface glycoproteins were incubated with 10ul (1ug/ul) allogeneic anti-Thy-1.1 or anti-Thy-1.2 monclonal antibody at 37°C for 30 minutes and followed by the addition of 30ul (1ug/ul) of rabbit anti-mouse IgM (affinity purified, Zymed Laboratories, Burlingame, CA).

The immunoprecipitate was obtained by centrifugation at 100,000xg for 60 minutes. Immunoprecipitation with xenogeneic anti-Thy-1.2 antibody was performed by incubation with 20ul (1ug/ul) xenogenic monoclonal anti-Thy-1.2 antibody followed by 30ul (1ug/ul) rabbit anti-rat IgG (Zymed Laboratories) followed by centrifugation as above.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis. Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed using discontinous 10% acrylamide gel in 0.2M tris/glycine buffer of pH7, essentially as described previously (21). Radiolabeled glycoproteins or immunoprecipitate were solubilized in 1mM tris/glycine buffer containing 1% SDS, 1% 2-mercaptoethanol 10% glycerol, and pyronin-Y tracking dye. Rabbit muscle myofibril protein was routinely run as a molecular weight standard (22). The gels with radioactive protein samples were cut into 2mm thick slices which were counted for radioactivity.

Non-equilibrium pH gradient electrophoresis. Resolution of gasic glycoproteins (including Thy-1) by non-equilibrium pH Oradient electrophoresis (NEPHGE) was performed as described by g'Farrell et al. (23). One dimensional electrophoresis was run in 91ass tubes (120mm x 3.0mm I.D.) with a gel mixture composed of 2.2M urea, 2% NP-40, 4% acrylamide/bisacrylamide (ratio 20:1) and 2%

Ampholines (BioRad, Richmond, CA). Ampholine pH3-10 was used bn the gels. Radioactive protein samples were dissolved in sample puffer composed of 9.5M urea, 2% NP-40, 1.6% pH5-7 ampholine, 0.4% 1H3-10 ampholine and 5% 2-mercaptoethanol. The samples were moaded and overlayed with 20ul overlay solution (8M urea and a Eixture of 0.8% pH 5-7 and 0.2% pH 3-10 ampholines). rlectrophoresis was for 3 hours at 500 volts. At the end of the tun, the gels were recovered by extrusion and cut into 1 or 2mm thick slices. The pH value or radioactivity of each slice was

RESULTS

Effect of Neuraminidase treatment on binding of Thy-1 glycoproteins with monoclonal anti-Thy-1 antibody.

Previous experiments suggested that removal of sialic acid residues from Thy-1 glycoproteins changed or decreased the expression of Thy-1.2 antigenicity (16). These conclusions were based on both a solid phase RIA and an immune response to Thy-1. Studies described in this report were designed to determine the mechanism of decrease of Thy-1 activity and to establish the role of sialic acid in Thy-1 expression. The neuraminidase sensitivity of Thy-1 was studied by immunoprecipitation of thymocyte glycoproteins after labeling with ^{125}I and solubilization in NP-40. Analysis of the whole NP-40 lysate by SDS-gel electrophoresis showed predominant labeling of T200 and Thy-1 (Figure 1A). Thy-1 was precipitated with allogeneic anti-Thy-1.2 (Figure 1B) or xenogeneic anti-Thy-1.2 (not shown). Both antibodies precipitated a glycoprotein with an apparent relative mass of 28 to 30,000 Da. Nase treatment of the NP-40 lysate followed by immunoprecipitation with allogeneic antibody resulted in a significant decrease in the precipitable material (Figure 1C). Extensive Nase treatment did not abolish this residual precipitable material. On the other hand precipitation of Nase treated glycoprotein with the xenogeneic anti-Thy-1.2 did not result in a significant

Figure 1. SDS-gel electrophoresis of radioiodinated C3H thymocyte NP-40 lysate and Thy-1.2 glycoprotein. (A) NP-40 cell lysate separated by 10% acrylamide gel electrophoresis. (B) Precipitation of sham-Nase treated cell lysate with monoclonal allogeneic anti-Thy-1.2 and anti-Thy-1.1 antibody (triangles). (C) Precipitation of Nase treated NP-40 lysate with anti-Thy-1.2. Myofibril protein standards used are indicated by arrows: myosin (220,000 Da), actin (45,000 Da), myosin light chain (24,000 Da), and the tracking dye.

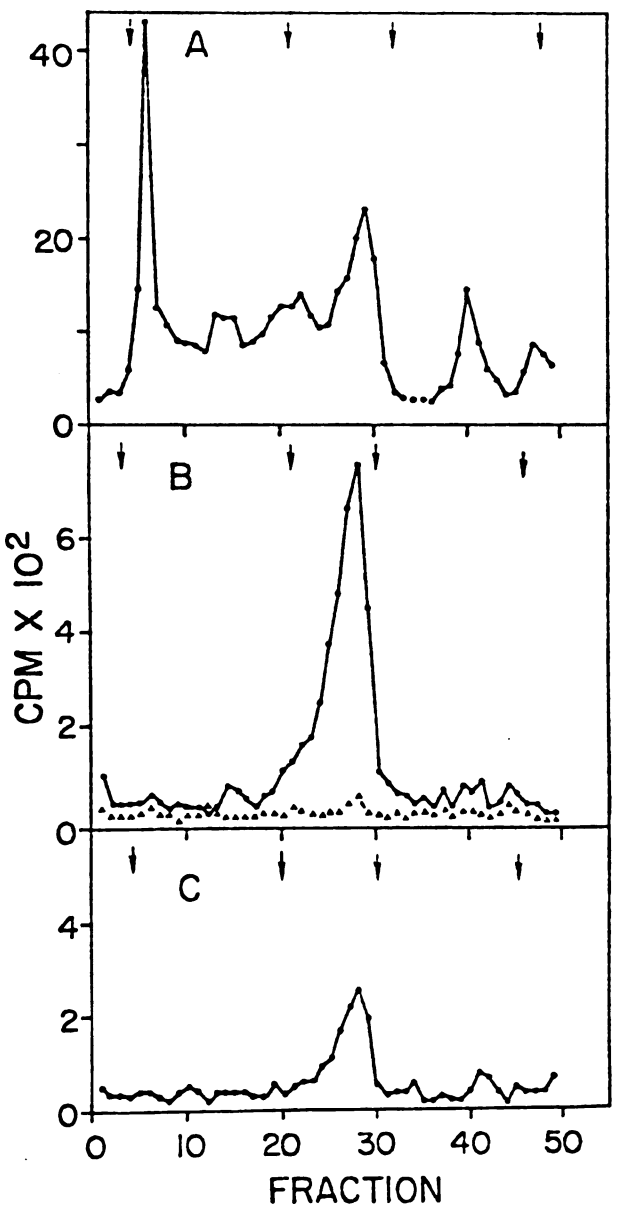

Figure 1.

reduction of Thy-1 precipitate (data not shown).

Characterization of neuraminidase treated thymocyte glycoproteins by non-equilibrium pH gradient electrophoresis. Previous reports using non-equilibrium pH gradient electrophoresis (NEPHGE) have shown that Thy-1 glycoprotein exhibits heterogeneity with respect to isoelectric point (13,14,15). Further it has been found that neuraminidase treatment decreased heterogeneity suggesting that at least part of the observed differences in pI was due to sialic acid residues. When C3H thymocyte glycoproteins were subjected to NEPHGE analysis, several species of basic glycoproteins were found toward the cathodal end of the gel (Figure 2A) which were identified by immunoprecipitation as Thy-1 glycoproteins (Figure 3). This pattern correlates with previous report that Thy-1 glycoproteins are among the more basic glycoproteins on thymocyte surface (15).

The effect of Nase treatment on the glycoproteins was first performed using the NP-40 lysate. After 2 hours of Nase incubation, only one major peak and two minor peaks chromatographed to the cathodal region of the gel (Figure 2B) and at 18 hours incubation, only one major and one more basic minor peak were observed (Figure 2C). This reduction of charge heterogeneity suggested that the heterogeneity of these glycoproteins was due to the variation in sialic acid content. It should be noted for purposes of comparison that

Figure 2. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) of radioiodinated cell lysate and Nase treated cell lysates. (A) Radioiodinated thymocyte NP-40 lysate with the pH gradient shown. (B) Lysate with 3 hours Nase treatment. (C) Lysate after 18 hours of Nase treatment. (D) Mixture of B and C. Gels are shown in standardized fashion for comparison with prviously published two dimensional gels and were sliced and numbered from the top (+anode) to the bottom (-cathode).

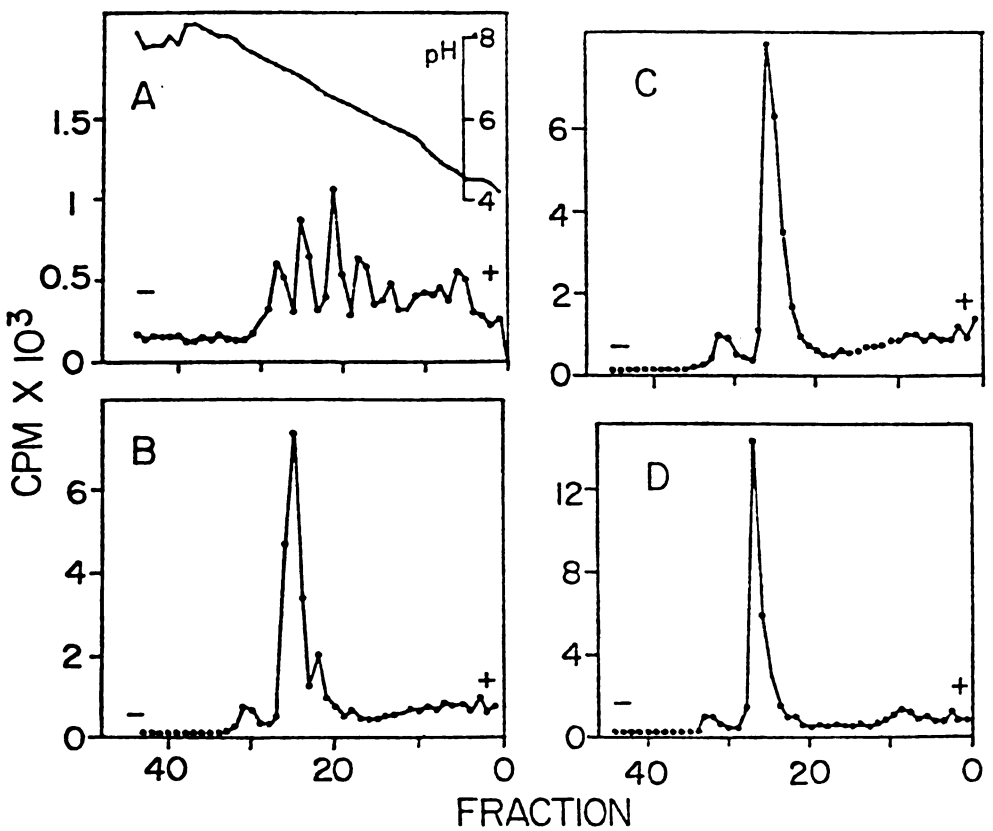

Figure 2.

Figure 3. NEPHGE analysis of allogeneic and xenogeneic immunoprecipitates. (A) C3H thymocyte NP-40 lysate immunoprecipitated with allogeneic anti-Thy-1.2. (B) Precipitation with xenogeneic anti-Thy-1.2. (C) Mixture of A and B The area of the xenoprecipitate is shaded in C to illustrate the mobility difference betwen allo and xenoprecipitates. Immunoprecipitates were analyzed by NEPHGE as shown in Figure 2 except that twice as many slices were cut to obtain the maximum resolution possible.

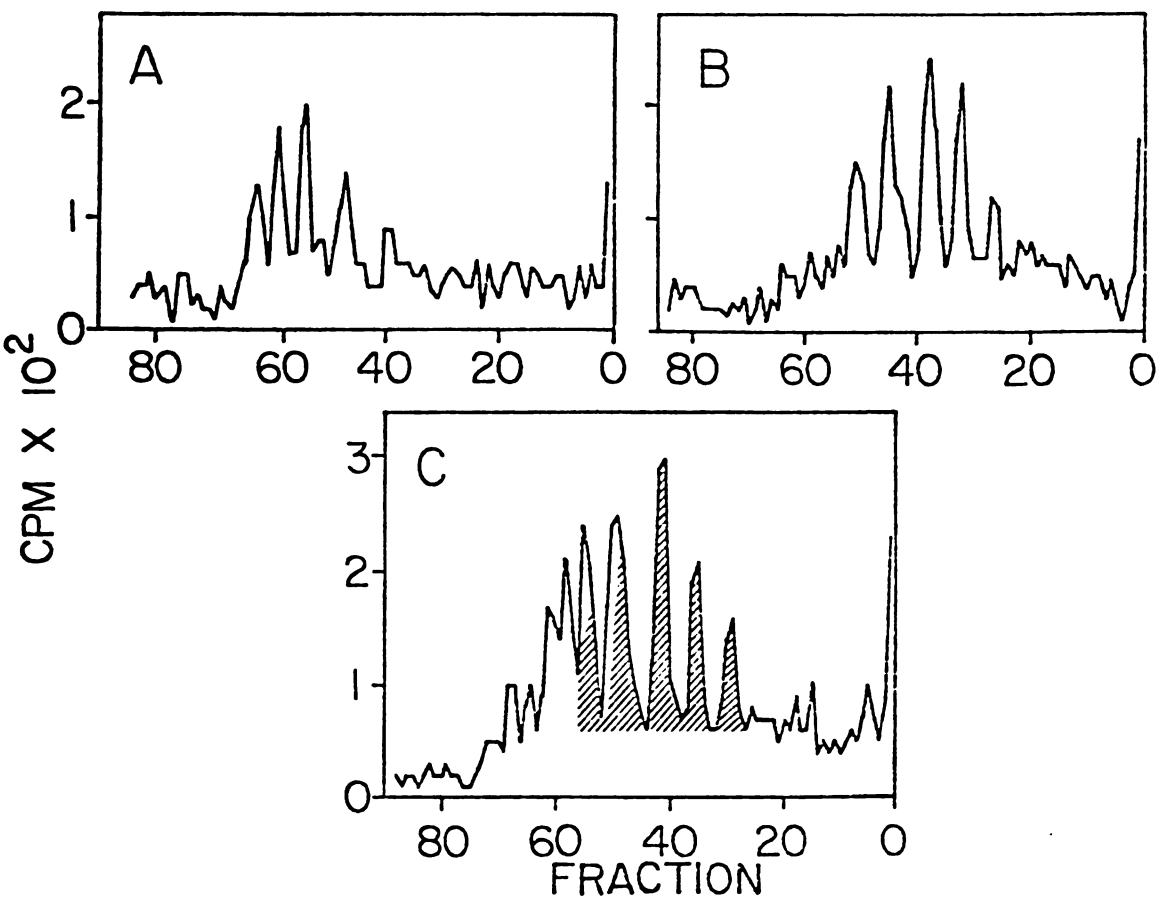

Figure 3.

the absolute position (within a fraction or two) on these gels could not be determined, therefore the 2 hour and the 18 hour Nase products were mixed and chromatographed on one gel (Figure 2D). Only one major peak and the more basic small peak was observed demonstrating that the 2 hour and 18 hour Nase products were identical in pI.

Immunoprecipitation of Nase treated Thy-1 glycoproteins after removal of sialic acid. Immunoprecipitation of NP-40 lysate of iodinated thymocytes was performed using monoclonal allogeneic (clone HO-13.4-22) and xenogeneic (clone 30-H12) anti-Thy-1.2 antibodies (Figure 3). Both of these antibodies were found by us and by others to be specific for Thy-1.2 by immunoprecipitation, cytotoxicity and fluoresent antibody analysis (13.19). The species of peaks appeared like the basic peaks observed in the whole NP-40 lysate (Figure 2A) and the five major components precipitated by the xenoantibody (Figure 3B) were in correspondance to previously published reports (13,14). There were however two differences noted between the monoclonal antibodies. First the xenogeneic antibody consistently precipitated three to five times as much radioactivity as the allogeneic antibody and second the components precipitated by the allogeneic antibody (Figure 3A) were more basic than the xeno-geneic precipitate. This difference in pI was illustrated by the observation of more than 8 components on a NEPHGE gel of the mixed

immunoprecipitates (Figure 3C). The peaks of the xenoprecipitate (shaded area) correspond to the anodal peaks in the mixture with considerable overlap among the central peaks in the mixture.

Immunoprecipitation of Nase treated NP-40 lysate using xenogeneic anti-Thy-1.2 and NEPHGE analysis resulted in only one major glyco-protein peak and a small amount of a more basic glycoprote (Figure in 4B). The absolute mobility of the precipitated peak appeared different from the Nase treated NP-40 lysate but mixing experiments confirmed their identity (not shown). Thus the xenoantibody precipi-tated two species of molecules the more basic being 10 to 20% of the main component. Immunoprecipitation with allogeneic anti-Thy-1.2 however demonstrated that this antibody preferentially precipitated only the minor more basic component (Figure 4C). The great enrichment of the more basic component suggest this antibody recognizes only this glycoprotein. The difference in mobility of the major protein and the minor basic protein was verified by mixing the allo-and the xeno-immunoprecipitates before NEPHGE anlysis (Figure 4D) which clearly shows different glycoproteins. Both the major and minor Nase products were precipitated by anti-Thy-1.2 antibodies as shown and were not precipitated with anti-Thy-1.1 antibodies thus indicating that they retained Thy-1.2 specificity. An overall decrease of about 75% in the amount of radioactivity

Figure 4. NEPHGE analysis of Nase treated glycoproteins after precipitation with allogeneic and xenogeneic anti-Thy-1.2 monoclonal antibodies. (A) Nase treated (18 hours) cell lysate. (B) Immunoprecipitation of Nase treated lysate with xenogeneic anti-Thy-1.2. (C) Immunoprecipitation with allogeneic anti-Thy-1.2. (D) Mixture of allogeneic and xenogeneic immunoprecipitates illustrates the mobility difference.

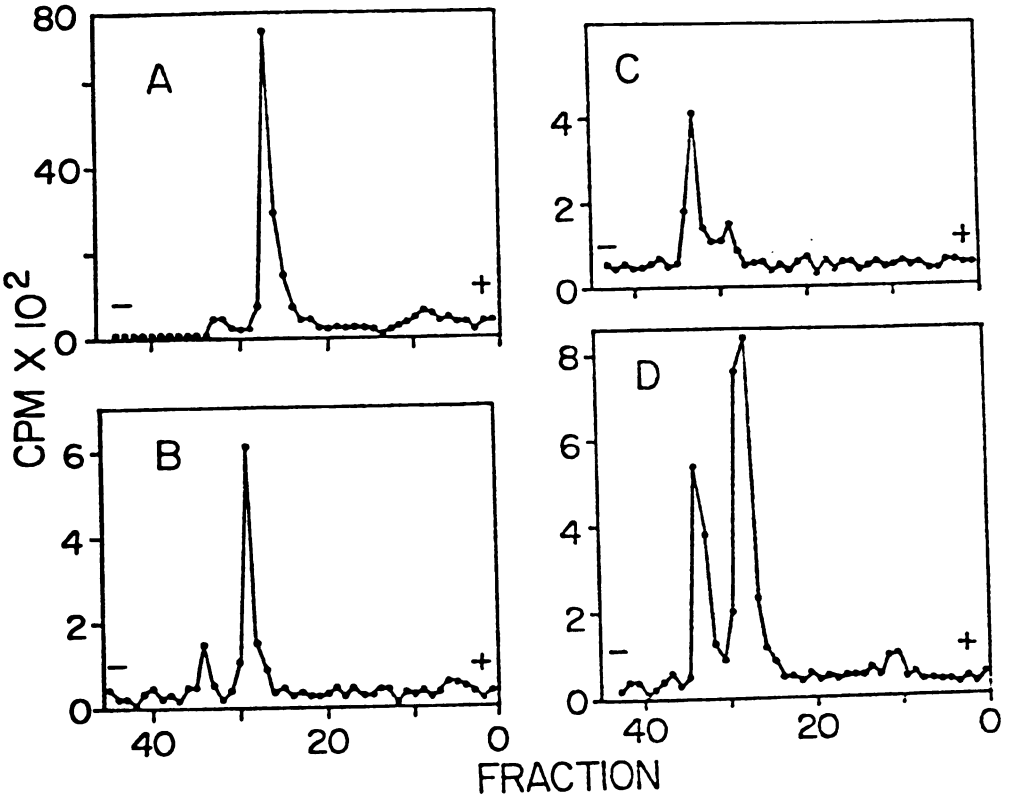

Figure 4.

precipitated with allogeneic anti-Thy-1.2 was observed, thus accounting for the loss of Thy-1.2 immunoprecipitate presented in Figure 1.

The possibility that the major glycoprotein could be converted to the more basic minor glycoprotein by further removal of sialic acid was tested by mild acid treatment of the NP-40 lysed thymocyte glycoproteins under conditions known to remove sialic acid (Figure 5A). The result demonstrated that: 1) sialic acid was removed from the glycoproteins resulting in a cathodal shift, 2) the predominant component formed was the major component as seen with Nase treatment, and 3) there was no apparent further conversion to the more basic minor component than observed with Nase treatment alone. The xenogeneic or allogeneic immunoprecipitates were also treated with mild acid to remove sialic acid residues and then analyzed by NEPHGE. The allogeneic immunoprecipitate contained the minor basic glycoprotein with only very small amounts of more acidic components (Figure 5C) and the xenogeneic immunoprecipitate contained the major glycoprotein with only small numbers of counts in the more basic area (Figure 5B). Incomplete hydrolysis was also observed in Figure 5B. Again the difference in mobility was confirmed by mixing and two separate species of glycoproteins were observed (Figure 5D). Thus immunoprecipitates of Nase treated NP-40 lysate exhibited essentially identical NEPHGE patterns to the

Figure 5. NEPHGE analysis of mild acid treated thymocyte NP-40 lysate and immunoprecipitates. (A) Radioiodinated C3H thymocyte NP-40 lysate after mild HCL treatment. (B) Mild acid treatment of Thy-1.2 glycoprotein in xenogeneic immunoprecipitate. (C) Mild acid treatment of Thy-1.2 glycoprotein in the allogeneic immunoprecipitate. (D) Mixture of mild acid treated Thy-1.2 glycoproteins from both xenogneic and allogeneic anti-Thy-1.2 immunoprecipitates (B and C).

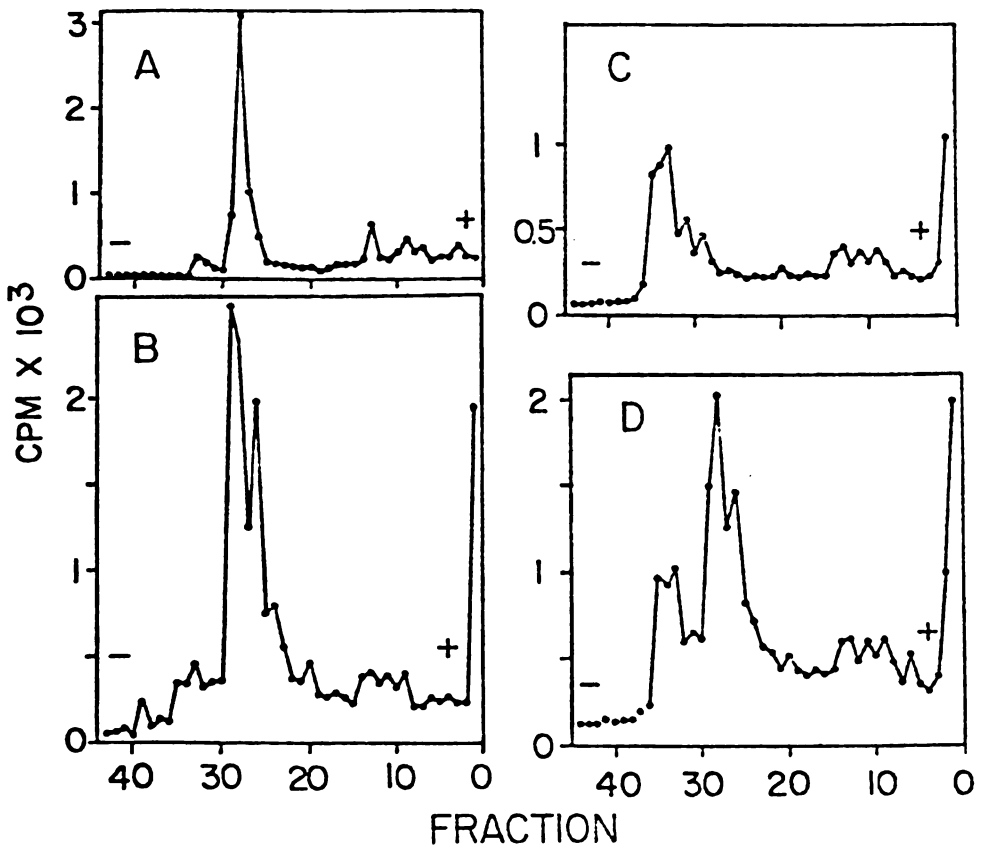

Figure 5.

chemically modified immunoprecipitates of NP-40 lysates without Nase treatment.

DISCUSSION

Monoclonal antibodies have been extremely useful in the characterization of subsets of lymphoid cell populations and in defining the specificity of antigenic systems. Anti-Thy-1 producing hybridomas have been prepared from allogeneic mouse immunizations (19,24) and from xenogeneic immunization of mouse cells (Thy-1.2) into rats which express Thy-1.1 (13). Some of the monoclonal antibodies have been shown to be allotype specific and react specifically with the Thy-1.1 or Thy-1.2 antigen and others have been shown to be directed to "framework" determinants and do not discriminate between Thy-1.1 and Thy-1.2. We have used the immunoglobulin products of two hydridoma clones, one allogeneic and one xenogeneic, which are specific for the Thy-1.2 determinant (13,19). A monoclonal anti-Thy-1.1 was used as a control antibody. These antibodies were used in experiments designed to assess the role of sialic acid in 1) the charge heterogeneity of Thy-1 glycoproteins and 2) in the expression of Thy-1 determinants. It has been previously proposed that sialic acid was important in expression of Thy-1 determinants (16) and that the sialic acid content of Thy-1 increases at different developmental stages (14).

Thy-1 glycoproteins have been shown to be complex in nature and to embody several unique features. SDS-gel electrophoresis of immuno-precipitated Thy-1 exhibits a

somewhat broad peak of about 25,000 to 30,000 Da. and Thy-1 derived from lymphoid cells consists of about 30% carbohydrate including sialic acid (4,11). The antigenic deter-minants of lymphoid Thy-1 are as yet unclear and proposals have been made that the determinants are carbohydrate or protein in nature. Rat brain Thy-1.1 has been sequenced and found to be devoid of a hydrophobic segment but contains a covalently bound lipid portion which is apparently responsible for membrane attachment (9). Fractionation of lymphocyte Thy-1 by nonequilibrium pH gradient electrophoresis resulted in the separation of five major components which differ in their sialic acid content (13,14,15). This has been illustrated by treatment of Thy-1 glycoprotein with neuraminidase followed by analysis by NEPHGE. The complex pattern of at least 5 components shifts toward the cathode resulting in one major component and one minor component at a more basic pH (Figure 2).

Previous reports (16) indicated that neuraminidase treatment of purified Thy-1 glycoprotein or thymocyte shed material resulted in a decrease of Thy-1 activity as assessed by the Thy-1 specific PFC assay (17). The reduction in Thy-1 activity after Nase treatment was corroborated by a solid phase radioimmunoassay using monoclonal allogeneic anti-Thy-1.2 (16). In contrast to these results Hoessli et al. (14,15) reported that Nase treatment of thymocytes did not significantly reduce

precipitation of Thy-1 with rabbit anti-mouse lymphocyte serum. We have also found that Nase treatment of thymocytes did not reduce the degree of binding of fluoresent anti-Thy-1.2 as assessed by flow cytometry (unpublished reuslts). We propose to further study this problem by use of different anti-Thy-1 antibodies in conjunction with the NEPHGE technique as described in this report.

The reduction in expression of Thy-1 antigen after Nase treatment was again verified using allogeneic anti-Thy-1.2 in a solid phase RIA (data not shown) and by immunoprecipitation with allogeneic anti-Thy-1.2 from NP-40 lysates before and after Nase treatment (Figure 1). Although we found a significant reduction in precipitable counts there was consistently a remaining precipitate which was apparently not affected by Nase treatment. This precipitate exhibited a molecular mass indistinguishable from Thy-1 before Nase treatment. Precipitation and SDS-gel analysis using a xenogeneic monoclonal anti-Thy-1.2 however resulted in a 25 to 30,000 Da glycoprotein, but there was not a decrease of Thy-1.2 precipitation after Nase treatment. This suggests that these two anti-Thy-1.2 clones distinguish different parts of the Thy-1.2 determinant. Since our previous studies used allogeneic systems it is probable that the decreases observed were below or at the sensitivity of the assay system (either the PFC assay or solid phase RIA). We now conclude that there is evidence

for a quantitative effect of sialic acid on the Thy-1.2 determinant but the overall activity depends on other protein or carbohydrate determinants. The effect of sialic acid may be to enhance the affinity of binding of antibody through charge or conformational effects. The reaction of the xenoantibody was not affected by Nase treatment indicating a lack of involvement of sialic acid in this recognition. This data does not preclude the possibility that these antibodies react with different parts or sites of the same antigenic determinants.

Nonequilibrium pH gradient electrophoresis of Thy-1.2 exhibited a pattern of five basic glycoproteins essentially identical to those previously reported (13,14,15). Neuraminidase treatment resulted in a shift toward the cathode as each species lost one or more sialic acid residues. Nase hydrolysis resulted in one major glycoprotein with a second small very basic component at the cathodal end. The very basic minor component comprised about 10 to 20% of the total Nase treated counts. Immunoprecipitation of thymocyte NP-40 lysate (before Nase treatment) with allogeneic and xenogeneic anti-Thy-1.2 showed that either antibody precipitated approximately five glycoproteins. The overall mobilities of the two precipitates were different showing that the two antibodies precipitated distinct families of molecules which were different in pI. The everlap of peaks between the allo- and

xenoprecipitate (Figure 3C) demonstrates that some of the peaks have similar pI. The resolution of the exact amount of overlap will require dual labeling of the precipitates with ^{3}H and ^{14}C.

The proposal that predominantly different species of glycoproteins were recognized by the allogeneic and xenogeneic antibodies was tested by immunoprecipitation after Nase treatment. The allogeneic antibody precipitated the most basic minor component with only very small amounts of the major Nase product which was probably precipitated non-specifically (Figure 4). The xenoantibody precipitated the major Nase component as well as an amount of the minor basic component similar to that present in the Nase treated lysate. This suggests that after Nase treatment the xenoantibody recognizes both forms of Thy-1.2 but the alloantibody only recognized the more basic minor component.

The possibility that the antibodies precipitate different molecules from the thymocyte surface was verified by mild HCl treatment of the respective immunoprecipitates. NEPHGE analysis of the HCl treated alloantibody precipitate resulted in only the more basic minor component with very small amounts of the other component which was considered to be a contaminant because of the high enrichment of the allo-precipitated component over the starting material (comparing Figure 5A and C). The HCl treated xenoprecipitate on the

other hand consisted of predominantly the major component as seen in Figure 5A and 4A. Again there was a small amount of the basic minor allo-recognized component present in about the same proportion as in the whole starting material. This suggests again that the xenoantibody precipitates both of the components. Removal of most or all of the sialic acid by Nase or HCl treatment resulted in identical cathodal shifts (to the same pI) of the two species of glycoproteins. Mild HCl treatment was effective in removing sialic acid (although some partial hydrolysis was seen, Figure 5B) and caused a cathodal shift which did not increase with further treatment. These two totally independent treatments, chemical and enzymatic resulted in the same minor and major basic glycoproteins which were recognized differently by the antibodies.

Thus we conclude that there are two different glycoproteins which carry Thy-1.2 activity which are present in thymocytes. The major component was recognized only by xenogeneic anti-Thy-1.2 and the minor component was recognized by both allogeneic anti-Thy-1.2 as well as the xenoantibodies. The distinction between the molecular determinants which both express a form of Thy-1.2 remains to be determined. At this time we have shown that the molecules differ in isoelectric point indicating that either the polypeptides are different or the molecules contain additional charged moieties (such as phosphate, sulfate or

amido differences). Both the allo-and xeno-precipitated species were found to exhibit a similar pattern of charge heterogeneity but at different pI. The relative quantity of the two components in the original cell lysate can be estimated by comparison of the relative radioactivity of the components found in NEPHGE analysis of the whole cell, Nase treated and HCl treated immunoprecipitates. Thus we have concluded that the minor (allo) component accounts for only about 10 to 20% of the major (xeno) component in the whole cell lysate.

It is possible that both the anti-Thy-1.2 antibodies are reacting with similar or related molecular determinants, for example a tetrapeptide or tetrasaccharide, which differ in only one residue. The difference between these may be recognized by the alloantibody but the antigenic determinants of both types may be sufficiently similar to be recognized by the xenoantibody. These two antibodies should be helpful in the elucidation of the exact structure of these determinants. The identification of two forms of Thy-1.2 with distinct reactions with monoclonal anti-Thy-1.2 antibodies suggests a degree of complexity and heterogeneity of the Thy-1 active glycoproteins which has not previously been appreciated. The role of Thy-1 in differentiation and its expression on differentiating T cells will require more complete and careful analysis utilizing both different types of monoclonal antibodies for analysis.

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Thy-1 glycoprotien : Radiolabeling of sialic acid and sensitivity to neuraminidase

FOOTNOTES

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[2] Studies performed in partial fulfillment of the Ph.D. Degree in Microbiology and Publich Health, Michigan State Universty.

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[4] Abbreviations: PFC, plaque forming cell; Nase, neuraminidase, Vc, Vibrio cholera; Mr, relative molecular mass; PBS, phosphate buffered saline; NP-40, Nonidet P-40; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; RIA, radioimmunoassay; NEPHGE, nonequilibrium pH gradient electrophoresis.

ABSTRACT

The sialic acid residues of thymocyte membrane glycoproteins were radiolabeled by mild oxidation with periodate and reduction with tritiated sodium borohydride. Radiolabeled sialic acid was identified by mild acid hydrolysis and by neuraminidase hydrolysis which removed 85 to 95% of radioactivity from isolated membrane glycoproteins. Radiolabeled membrane glycoproteins were solubilized with NP-40 and studied by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Four major radiolabeled glycoproteins were identified, with approximate Mr of 100,000, 45,000, 29,000 and less than 20,000 Da, and several weakly labeled glycoproteins were also identified. Immunoprecipitation of radiolabeled glycoproteins with two different monoclonal anti-Thy-1.2 antibodies precipitated a glycoprotein of approximately 27 to 29,000 Da indicating that Thy-1 glycoprotein was labeled by this procedure. Neuraminidase treatment of intact cells followed by labeling of cell surface sialic acid indicated a partial degradation (about 50% in one hour) of the cell surface glycoproteins. The sialic acid residues of the glycoproteins solubilized by NP-40 were hydrolyzed almost completely after 18 hours of neuraminidase treatment. This demonstrats that the membrane glycoproteins, including Thy-1, were sensitive to neuraminidase both in the intact membrane and in purified

form. Immunoprecipitation with xenogeneic anti-Thy-1.2 followed by analysis by non-equilibrium pH gradient electrophoresis (NEPHGE) resulted in a pattern of five components with charge heterogeneity. The sialic acid labeled components compared favorably with the pattern obtained with radioiodinated Thy-1.2. Increasing amounts of tritium relative to ^{125}I of the Thy-1 glycoproteins reflected the increased ratio of sialic acid to the ^{125}I labeled polypeptide. Neuraminidase treatment of the Thy-1 demonstrated a decrease in overall radioactivity a cathodal shift expected for removal of sialic acid. The labeling of Thy-1 with periodate/borohydride should be useful in the analysis of Thy-1 oligosaccharides.

INTRODUCTION

Thy-1 antigen was first identified by Reif and Allen (1966) as a murine differentiation antigen of thymocytes and brain. Thy-1 has been mapped on chromosome 9 and consists of two alleles, Thy-1^a and Thy-1^b, which code for Thy-1.1 and Thy 1.2 serotypes. Thy-1.1 has been identified in AKR, A/Thy-1.1 and RF mice and the Thy-1.2 specificity has been found in the majority of other strains of mice. Rats have been found to exhibit only the Thy-1.1 serotype. Mouse thymocytes, T lymphocytes and brain contain the highest concentrations of Thy-1 antigen, with smaller amounts in epidermis, fibroblasts and other tissues (Scheid, et al., 1972; Stern, 1973; William et al. 1977).

Thy-1 glycoproteins have been characterized by SDS gel electrophoresis and exhibit a Mr of 25,000 to 30,000 Da with charge heterogeneity and some Mr variation, probably due to different degree of glycosylation (Ledbetter & Herzenberg, 1979; Hoesseli et al., 1980). Chemical analysis has shown that Thy-1 glycoprotein contains about 30% carbohydrate (including sialic acid) and the amino acid sequence of rat brain Thy-1.1 has also been reported (Campbell et al., 1981). The presence of Thy-1 active glycolipids in mouse brain, lymphocytes, and lymphoma cells has also been reported (Wang, et al., 1978; Kato, et al., 1979). The Thy-1 specificity of the glycolipids was measured by a Thy-1 plaque forming cell assay and was found to be identical to

the glycoprotein preparations in the same assay. Biochemical studies, including sensitivity to neuraminidase and mild acid, have indicated that Thy-1 glycolipids have the propoerties of gangliosides (sialic acid containing glycolipids).

The activity of Thy-1.2 active glycoprotein has been found to decrease after neuraminidase treatment when the activity was tested with allogeneic anti-Thy-1.2 (Wang, et al., 1980). On the other hand Thy-1.2 activity appears unchanged after Nase treatment when assesed with xenogeneic anti-Thy-1.2 (Wang, et al., 1982). The relationship of Thy-1 active glycoproteins and gangliosides is as yet unclear and the chemical nature of the Thy-1.1 and Thy-1.2 antigenic determinants is not known. The discovery of Thy-1 active glycolipids suggests that the Thy-1 determinants are carbohydrate in nature but others have proposed that the determinants are protein in nature because extensive protease digestion destroys the activity (Williams et al., 1977).

The present experiments were designed to assess the role of sialic acid in the heterogeneity of Thy-1 by the introduction of tritium into sialic acid of Thy-1 and other membrane glycoprotein followed by examination of their neuraminidase sensitivity in both intact cells and in isolated form.

MATERIALS AND METHODS

Radiolabeling of thymocyte surface sialoproteins. The method of radiolabeling cell surface sialoproteins was described by Gahmberg & Anderson (1977). We have used this method with some modifications to radiolabel mouse thymocytes obtained from AKR/J(Thy-1.1) and C3H/HeJ(Thy-1.2) mice (Jackson Laboratory , Bar Harbor, ME). Thymocyte suspensions ($5x10^7$ cells) were washed with phosphate buffered saline (PBS) 3 times and then treated with 5mM $NaIO_4$ at 4°C for 20 minutes. The reaction was stopped by adding 0.2ml PBS containing 0.2M glycerol and cells were washed three times in PBS. The buffering capacity of the cell suspension was increased to 0.5M phosphate buffer to minimize cell lysis by the basic borohydride. One mCi of $[^3H]NaBH_4$ (specific activity 9.5Ci/mmole, Amersham Co., Arlington Hgts, IL) was added to the cell suspension and incubated at room temperature for 30 minutes. The suspension was then washed three times with PBS and the viability of the cells determined by trypan blue exclusion was usually greater than 90%. Treatment of control cells (no $NaIO_4$) with 1mCi $[^3H]NaBH_4$ generally resulted in less than 10% of radioactivity incorporated compared to periodate treated cells. After cell surface labeling, a crude membrane fraction was prepared by use of a Dounce Homogenizer (Kontes Co., Vineland, NJ) followed by centrifugation for one hour at 10,000xg to remove

mitochondria and nuclei. The supernatant was then centrifuged at 100,000xg for 60 minutes to pellet membraneous fragments. The majority of Thy-1 antigenic activity was recovered in the crude membrane preparation (Schwarz et al., 1980).

Radioiodination of cell surface glycoproteins was performed as described previously (Marchalonis, et al., 1971) with minor modifications. Briefly $7x10^7$ thymocytes were incubated with 120ul lactoperoxidase (1mg/ml in PBS Sigma, St. Louis, MO), 500uCi of carrier free $Na^{125}I$ (New England Nuclear, Boston, MA) and 15ul of 10mM H_2O_2. The reaction was stopped after 5 minutes by washing, and membranes were prepared as described above. The membrane preparation was solubilized with 1% NP-40 at 4°C for 18 hours. Insoluble cell debri was removed by centrifugation at 100,000xg for 60 minutes. The NP-40 solubilized membrane preparation was then used for immunoprecipitation.

Immunoprecipitation of Thy-1 glycoprotein. Three sources of monoclonal anti-Thy-1 antibody were used. Allogeneic IgM anti-Thy-1.1 (clone HO-22.1.6) and IgM anti-Thy-1.2 (clone HO-13.4-2.2) antibodies were obtained from New England Nuclear (Boston, MA) in the form of ascites fluid. These were produced by injection of AKR thymocytes into (Balb/c x Balb.k) F_1 mice and by injection of C_3H thymocytes into AKR mice (Marshak-Rothstein, et al., 1979). Xenogeneic IgG anti-Thy-1.2 (clone 30-H12) antibody was

purchased from Becton Dickenson (Sunnyvale, CA). Hybridoma line 30H-12 (Ledbetter & Herzenberg et 1979) was also obtained from the Cell Line Distribution Center at the Salk Institute. This cell line resulted from injection of mouse thymocytes into Lou/WsI.M rats (Thy-1.1). Supernatant fluids were routinely collected by culturing cells in the inner chamber of Marbrook vessels (1ml) with 10ml of culture medium and antibody was perpared by $(NH_4)_2SO_4$ precipitation. Radiolabeled membrane derived from 5×10^6 thymocytes was treated with 300ul PBS with 1% NP-40 for 18 hours at 4°C. The suspension was cleared by centrifugation at 150,000xg for 30 minutes. The supernatant was then cleared of non-specific precipitable material by the addition of an antigen and its respective antiserum (usually normal rat serum followed by rabbit anti-rat serum) followed by centrifugation at 150,000xg for 30 minutes. Immunoprecipitation was initiated by the addition of 10ul allogeneic monoclonal antibody (1ug/ul) or 10ul (1ug/ul) xenogeneic monoclonal antibody and incubated at 37°C for 30 minutes. Rabbit anti-mouse Ig or anti-rat Ig (30ul affinity purified, 1ug/ul, from Zymed Laboratories, Burlingame, CA) was added and incubated for an additional 20 minutes. The resulting precipitate was separated by ultracentrifugation at 150,000xg for 60 minutes. The supernatant fluid was removed and the precipitate was solubilized and denatured with SDS-electrophoresis or NEPHGE sample buffer.

Sodium dodecylsulfate polyacrylamide gel electrophoresis. Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed using discontinuous acrylamide gel in 0.2M tris-glycine buffer pH 7, essentially as described previously (Laemmli, 1970). The stacking gel consisted of 4.6% acrylamide, tris-glycine buffer pH 7 and the separating gel consisted of 10% acrylamide (acrylamide/bis ratio 100:1). Radiolabeled membrane glycoprotein or immunoprecipitate was solubilized in 5mM Tris/glycine buffer containing 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and Pyronin-Y tracking dye; and heated at 100°C for 10 minutes. Rabbit muscle myofibril protein was routinely run as a standard(Porzio & Pearson, 1977). The gels with radiolabeled protein sample were cut into 2mm slices which were placed into glass scintilation vials with 10ml toluene cocktail (POP 5.5g/liter, POPOP 0.1g/liter) containing 3% Protosol (New England Nuclear) and 10% H_2O. The vials were capped and brought to 60°C for 24 hours and then counted. The recovery of tritium applied on the gel was usually about 80%. The estimated molecular weight of each radioactive glycoprotein species was obtained by comparison to the myofibril protein standard.

Nonequilibrium pH Gradient Electrophoresis. Resolution of basic glycoproteins (including Thy-1) by non-equilibrium pH gradient electrophoresis (NEPHGE) was performed as described by O'Farrell et al. (1977). One dimensional

electrophoresis was run in (120mmx2.0mm I.D.) glass tubes with a gel mixture composed of 9.2M urea, 2% NP-40, 4% acrylamide/bisacrylamide (ratio 20:1) and 2% ampholines (BioRad, Richmond, CA). Radioactive protein samples were dissolved in sample buffer composed of 9.5M urea, 2% NP-40, 1.6% pH5-7 ampholine, 0.4% pH3-10 ampholine and 5% 2-mercaptoethanel. The samples were loaded and overlayed with 20ul overlay solution (8M urea and a mixture of 0.8% pH 5-7 and 0.2% pH 3-10 ampholines). Electrophoresis was for 3 hours at 500 volts. The gels were recovered by extrusion and cut into 2mm thick slices. The pH value or radioactivity of each slice was then determined as described above.

Removal of radiolabeled sialic acid from Thy-1 glycoprotein.

The extent of radiolabeling of sialic acid was determined by using mild acidic conditions which selectively remove sialic acid. Radiolabeled Thy-1 glycoprotein was isolated by elution from SDS-gel slices into PBS and then treated with 0.1N HCl at 90°C for 30 minutes (Esselman, et al., 1972; Wang, et al., 1982). The mixture was chilled, adjusted to neutral pH by addition of 0.1N NaOH and was chromatographed on a Sephadex G-50 column (1 x 50cm) eluted with 0.1M NH_4HCO_3. Fractions were collected and radioactivity was monitored. Vibrio cholera Neuraminidase (Calbiochem-Behring, La Jolla CA.) was used to treat thymocytes for

various periods of time at the concentration of 0.1 International Units(IU)/10^8 cells. After treatment, cells were washed 3 times with PBS and radiolabeled with periodate/borohydride as described in previous sections. Radiolabeled glycoproteins were then isolated by NP-40 solubilization and chromatographed by SDS-gel eletrophoresis. Control cells received heat-inactivated neuramindase and were incubated under the same conditions. Radiolabeled, purified glycoproteins were treated with neuraminidase at 0.3 IU/10^5cpm for various period of time. The reaction was terminated at 100°C for 10 minutes and the samples were precipitated from 75% ethanol at -20°C and chromatographed by SDS-gel eletrophoresis.

RESULTS

Radiolabeling of sialic acid of membrane glycoproteins.

The effect of mild periodate oxidation and sodium borohydride reduction on Thy-1 activity was initially assesed by treatment of thymocyte membrane complexes bound to microtiter plates (Schwarz et al., 1980). The binding of monoclonal allogeneic [^{125}I]anti-Thy-1.2 to the complexes was assesed before and after periodate oxidation and borohydride reduction. Expression of Thy-1 activity was determined to be essentially unchanged by this treatment: untreated wells bound 1500$\pm$200 CPM of (^{125}I) antibody; treated wells bound 1400$\pm$250 CPM; and control wells bound 200 $\pm$ 35 CPM.

The sialic acid portion of membrane glycoproteins of mouse thymocytes was radiolabeled by oxidation of intact cells with 5mM $NaIO_4$ followed by reduction with 1mCi of high specific activity [^{3}H]$NaBH_4$. The conditions used ensured that sialic acid moieties on glycoproteins and glycolipids would be preferentially oxidized with periodate leaving other cell components relatively unchanged (Gahmberg & Anderson, 1977). Possible cell lysis by basic $NaBH_4$ was avoided by increasing the buffering capacity of the cell suspension to 0.5M phosphate buffered isotonic saline. Higher than 90% viability was found after oxidation and reduction was complete. The crude membrane preparation of labeled cells was dissolved and denatured in sample buffer

Figure 1. SDS-gel electrophoresis of periodate/borohydride labeled C3H thymocyte glycoproteins and Thy-1 glycoproteins. (A) NP-40 lysate of radiolabeled C3H thymocyte separated by 10% acrylamide gel, (B) immunoprecipitate from radiolabeled cell lysate with allogeneic anti-Thy-1.2 or anti-Thy-1.1 (triangle) monolconal antibody;and (C) immunoprecipitate from cell lysate with xenogeneic anti-Thy-1.2 monoclonal antibody. Myofibril protein was used as standards and marked by arrows: a) alpha-actinin (102,00Da), b) Actin (45,000 Da), c) Tropomyosin (35,000Da), d) myosin light chain (24,000 Da) and e) dye front.

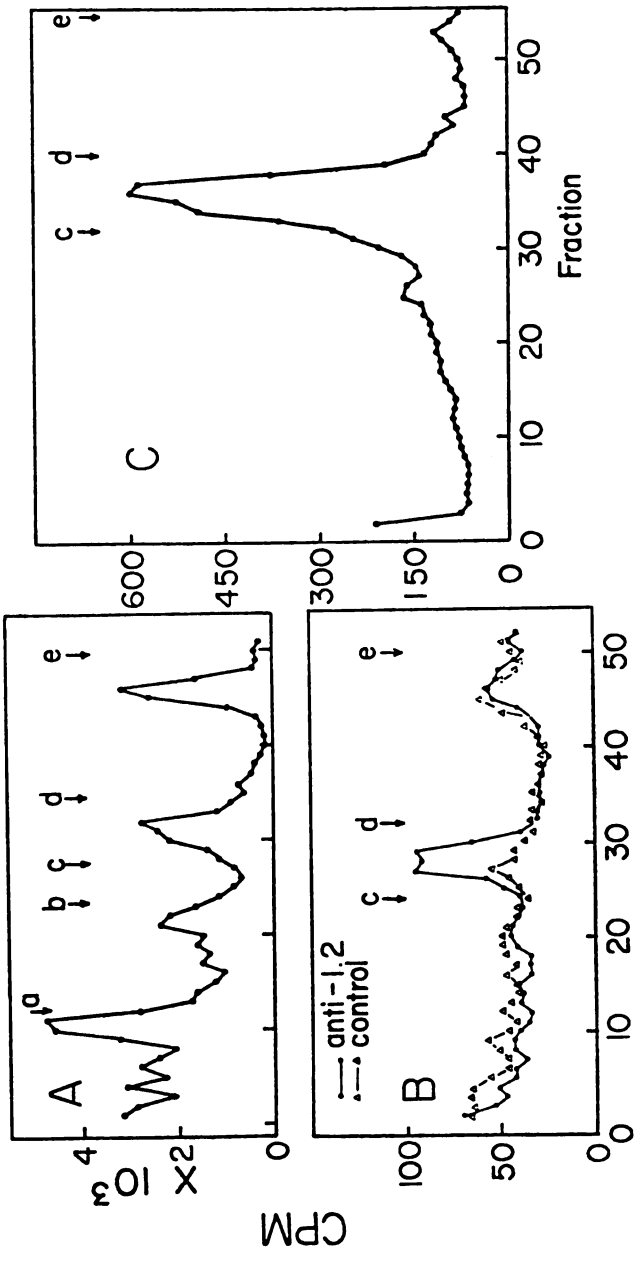

Figure 1

and chromatographed by SDS gel electrophoresis (Figure 1A). Analysis of C3H thymocytes allowed the identfication of four major glycoproteins with approximate Mr of 45,000, 100,000, 29,000 and less than 20,000 Da.

Immunoprecipitation of Thy-1 glycoprotein with allogeneic and xenogeneic monoclonal antibody. Verification of the tritium labeling of Thy-1 active glycoprotein was performed by specific immunoprecipitation. Three antibodies were used: monoclonal allogeneic anti-Thy-1.1 and anti-Thy-1.2 and monoclonal xenogeneic anti-Thy-1.2. For immunoprecipitation, the crude membrane preparation of radiolabeled C3H thymocytes was solubilized with 1% NP-40 and antibodies were added as described in the Materials and Methods. The precipitated glycoproteins were then chromatographed by SDS-gel electrophoresis. Immunoprecipitation of xenogeneic anti-Thy-1.2 resulted in precipitation of a 27 to 29,000 Da glycoprotein (Figure 1C). The labeling process did not alter the Thy-1 antigenicity as judged by binding of xenogeneic anti-Thy-1 antisera because the efficiency of precipitation was approximately 80% of labeled glycoproteins. The labeled membrane glycoproteins were also treated with either allogeneic anti-Thy-1.2 or anti-Thy-1.1 (as a control) and chromatographed by SDS-gel electrophoresis. Anti-Thy-1.2 precipitated a glycoprotein of approximately 29,000 Da (Figure 1B). This glycoprotein was not precipitated with the anti-Thy-1.1 antiserum. The

efficiency of precipitation with allogeneic anti-Thy-1.2 was only about 20% of the xenogeneic anti-Thy-1.2.

Neuraminidase and acid sensitivity of thymocyte sialoglycoproteins. The position of the tritium label on the Thy-1 glycoprotein was verified by release of sialic acid using mild acid and neuraminidase hydrolysis. The 29,000 Da Thy-1 active different in the Material and Methods into PBS and was treated with
0.1N HCl using conditions which perferentially remove sialic acid (Esselman et al., 1972). The glycoprotein was then chromatographed on a Sephadex G-50 column. The radioactivity associated with Thy-1 eluted in the void volume as micelles. After mild acid hydrolysis about 90% of the radioactivity eluted in the included column volume with sialic acid (Figure 2). Neuraminidase treatment was also found to remove approximately 90% of the incorporated tritium from labeled Thy-1 glycoprotein using the same assay system. It was concluded therefore that the sialic acid moiety was the primary site for incorporation of tritium in the glycoprotein molecule.

NP-40 solubilized thymocyte glycoproteins were treated with neuraminidase to determine their relative sensitivity to the enzyme. Thymocytes were labeled, solubilized and treated with neuraminidase, and at varying periods of time aloquots were removed for SDS-gel electrophoresis (Figure 3). After 0.5 hours and 4 hours of treatment all four

Figure 2. Removal of tritium labeled sialic residues with mild acid hydrolysis. Periodate/borohydride radiolabeled Thy-1 glycoprotein was obtained by immunoprecipitation with xenogeneic anti-Thy-1.2 monolconal antibody and preparative SDS gel elctrophoresis. The Thy-1 was chromatographed on a Sephadex G-50 column (open cirlce). One part was treated with 0.1N HCl at 55°C for 2 hours (closed circles).

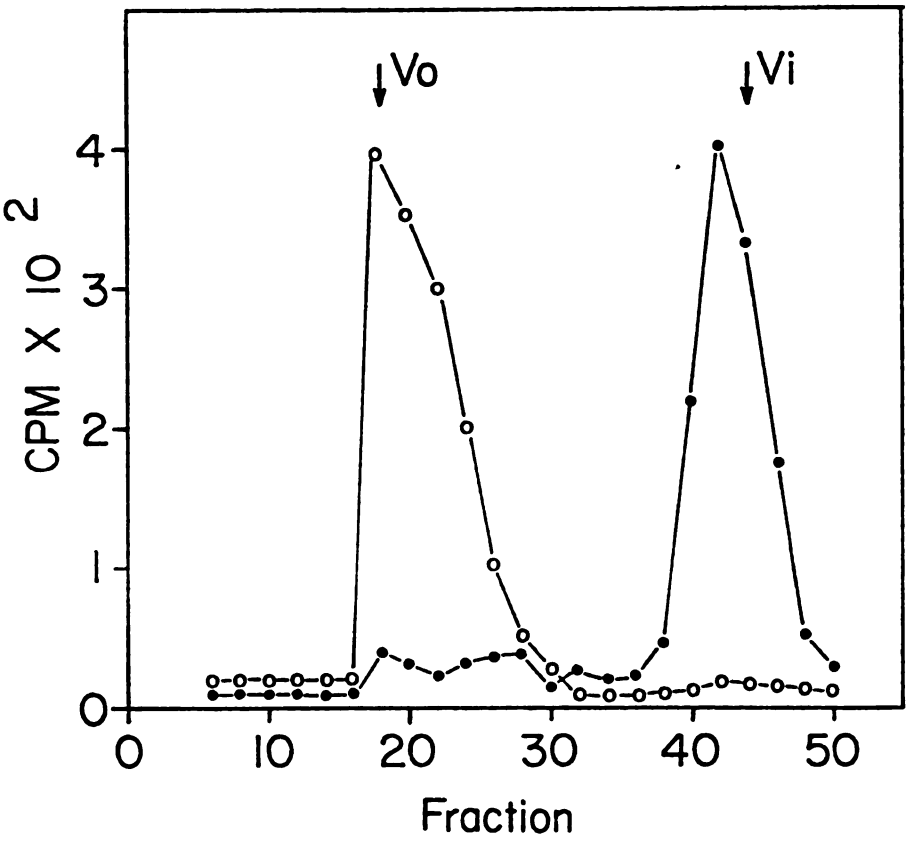

Figure 2

Figure 3. Vibrio cholera neuraminidase treatment of periodate/borohydride radiolabeled C3H glycoproteins or C3H thymocytes prior to radiolabeling. (A-C) 1% NP-40 lysate of radiolabeled C3H thymocytes containing $2X10^5$ CPM treated with 0.1IU nueraminidase for the indicated period of time; (D) 10^8 C3H thymocytes treated with heat inactivated neuraminidase (D) or with active neuramindase (E) for 30 minutes at 37°C before periodate/borohydride labeling. Samples were analyzed by SDS-gel electrophoresis. Glycoprotein mobilities were comparable to Figure 1 and the dye front is indicated by an arrow.

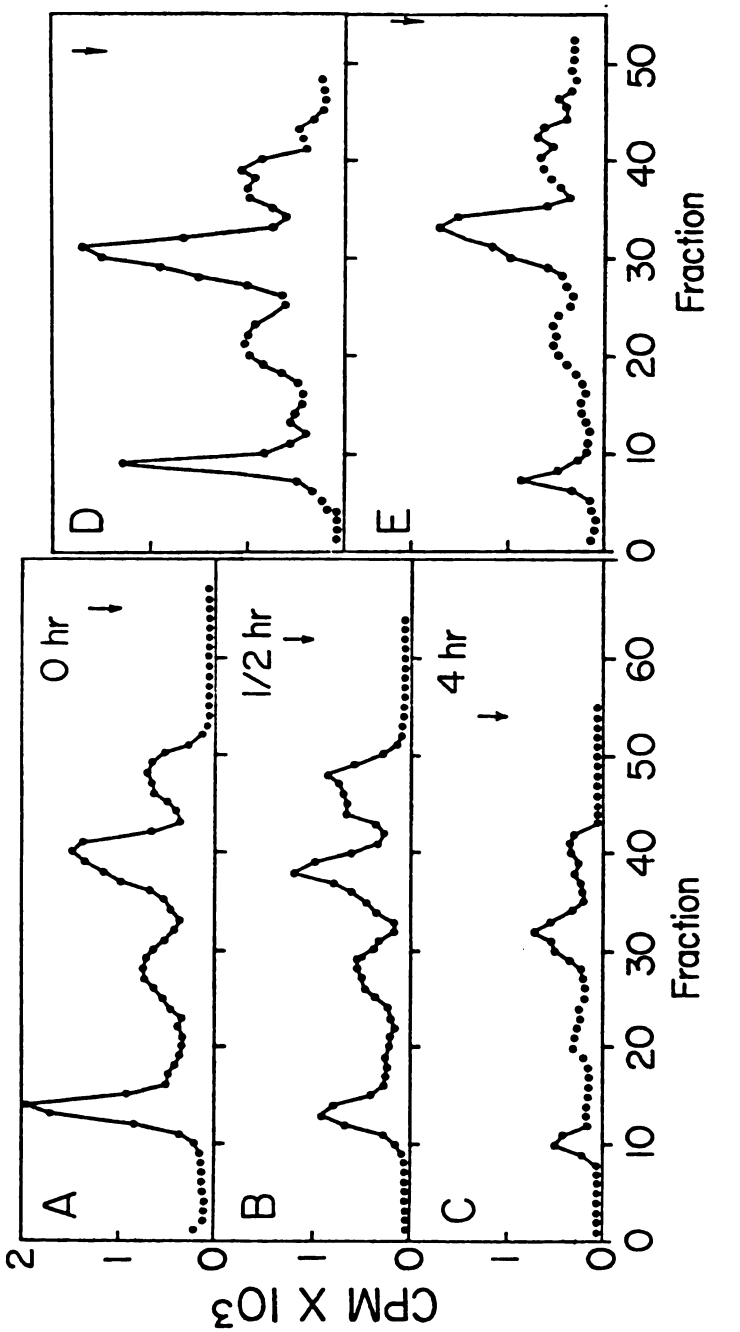

Figure 3

glycoproteins were decreased but the 100,000 Da glycoprotein consistently appeared to decrease at a greater rate than the other three (Figure 3B and C). After 16 hours of treatment essentially all of the sialic acid was removed from the four components.

To determine the degree of expression of Thy-1 on the cell membrane, thymocytes were treated with neuraminidase before labeling with periodate/borohydride. The membranes were then solubilized and analyzed by SDS-gel electrophoresis (Figure 3D and E). The sialoglycoproteins were all degraded after 1 hour indicating that the sialic acid residues of each was available to neuraminidase. The 100,000 Da glycoprotein again appeared to be somewhat more labile than Thy-1 to neuraminidase. Longer neuraminidase incubation preiods were not used because of increased cell death after about 1.5 hours of incubation.

The presence of Thy-1.2 determinants on partially Nase degraded Thy-1 glycoprotein was shown because Thy-1.2 was still precipitable after Nase treatment (not shown). After accounting for loss of tritiated sialic acid there was a 75% decrease in the amount of precipitate using allogeneic anti-Thy-1.2 but no decrease in precipitability was observed with the xenogeneic anti-Thy-1.2. The exact magnitude of the decrease of Thy-1 activity was not completely measurable using the periodate labeling technique described in this report because removal of the radioactive sialic acid

resulted in loss of radioactivity from the glycoprotein.

Analysis of sialic acid labeled Thy-1.2 by nonequilibrium pH gradient electrophoresis. The NP-40 lysate of thymocytes labeled by periodate/borohydride treatment was immunoprecipitated using xenogeneic anti-Thy-1.2 and analyzed by nonequilibrium pH gradient electrophoresis (NEPHGE). The series of about five major peaks observed were compared to the peaks obtained by identical precipitation with iodinated Thy-1.2 (Figure 4). In progressing from the basic pH to the acidic region of the gel the ratio of ^{3}H to ^{125}I for each peak increased as expected because of the increased number of sialic acid moities per polypeptide chain. Thus the ratio $^{3}H/^{125}I$ was largest in the most acidic Thy-1.2 glycoproteins (Figure 4, fraction 15).

Neuraminidase treatment of Thy-1.2 was performed using the whole cell lysate because Nase was found to be ineffective on immunoprecipitates. This was also possible because the very basic glycoproteins present in the NP-40 lysate (Figure 5A) were essentially identical to those in the Thy-1.2 immunoprecipitate. Thus the pattern of proteins in Figure 5A were identified as Thy-1.2 glycoproteins. The NEPHGE gel pattern obtained by partial neuraminidase hydrolysis of Thy-1.2 indicated that removal of sialic acid decreased the radioactivity associated with the Thy-1.2 glycoproteins while increasing the pI of these components

Figure 4. Comparison of NEPHGE analysis of periodate/borohydride radiolabeled Thy-1 glycoproteins and radioiodinated Thy-1 glycoproteins. C3H thymocytes were radiolabeled with either periodate/borohydride or lactoperoxidase-catalyzed iodination as described in Materials and Methods. The NP-40 lysate of radiolabeled thymocytes was immunoprecipitated with xenogeneic anti-Thy-1.2 monoclonal antibody and the immunoprecipitate was analyzed by NEPHGE. The upper panel shows the pH gradient formed in the gel. The middle panel shows NEPHGE analysis of tritium radiolabeled Thy-1 glycoprotein and the lower panel shows pattern of radioiodinated Thy-1 glycoprotein. The gels were sloced and numbered from the top, and are oriented in the standard fashion for comparison to two dimensional gels.

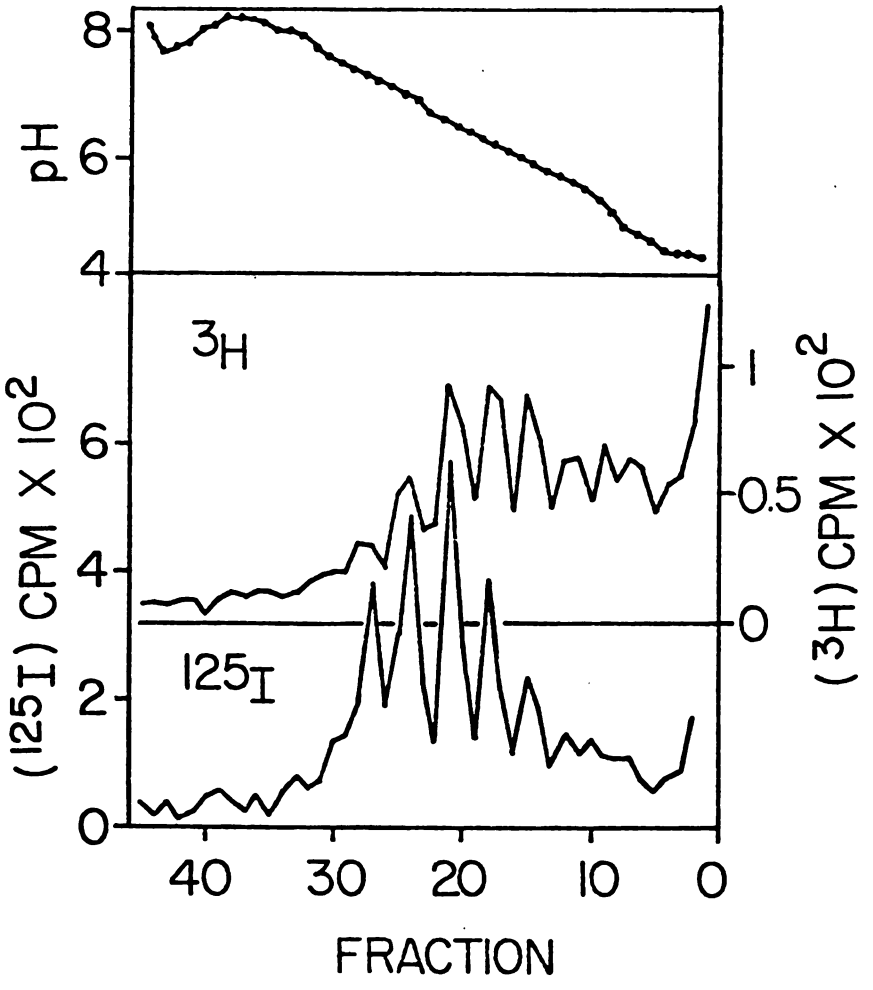
pH
8
6
4
3H
125I
(125I) CPM X 10²
(3H) CPM X 10²
6
4
2
0
1
0.5
0
40
30
20
10
0
FRACTION

Figure 4

(Figure 5B). After 60 minutes of Nase treatment the only radioactivity remaining was associated with the most basic component observed (Figure 5C). Hydrolysis of Thy-1 with Nase for 18 hours (or HCl treatment) completely removed sialic acid and little radioactivity was observed in the gels. The basic component produced by Nase treatment comigrated with the iodinated component formed by Nase treatment of iodinated Thy-1.2 glycoprotein prepared as previously described (Wang et al., 1982). The major Nase product was immunoprecipitable with xenogeneic anti-Thy-1.2 but the allogeneic anti-Thy-1.2 did not precipitate sufficient radioactivity for NEPHGE analysis. Partial Nase hydrolysis did not change the apparent Mr of the glycoprotein as judged by SDS-gel electrophoresis.

Figure 5. NEPHGE analysis of periodate/borohydride radiolabeled C3H thymocyte glycoproteins treated with neuraminidase. C3H thymocytes $5x10^7$ were treated with neuraminidase for 0 minutes (A), 30 minutes (B) and 60 minutes (C). After treatment, cells were labeled with periodate/borohydride, lysed with 1% NP-40, and analyzed by NPEHGE. Gels were sliced and radioactivity was determined. Nase treated ^{125}I labeled thymocyte glycoproteins were mixed with sample (C) and chromatographed in same gel (dashed line)

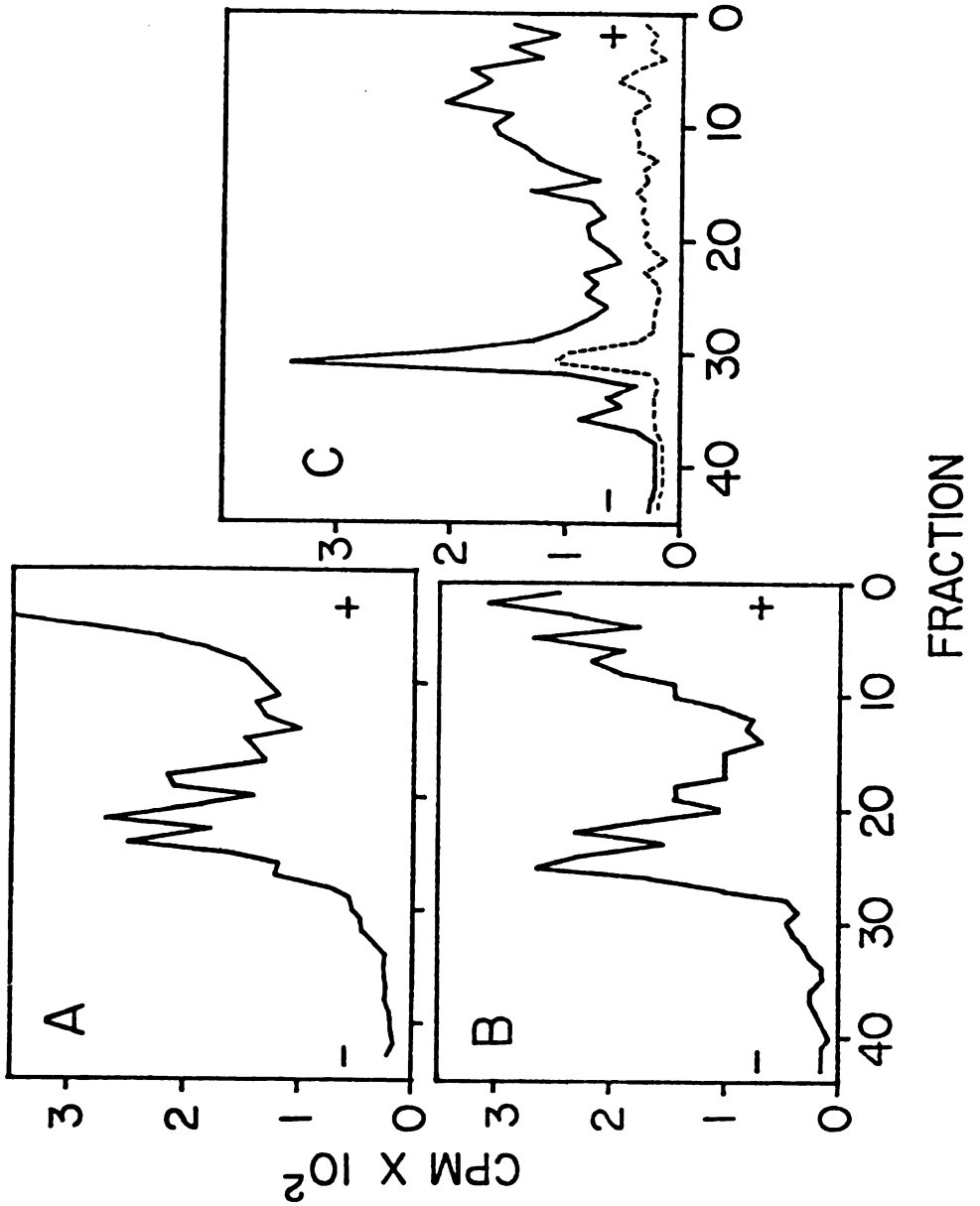

Figure 5

DISCUSSION

Thy-1 glycoprotein antigen has been found to be complex molecule exhibiting charge and size beterogeneity both as well as forms which may include glycolipids. Several groups have isolated Thy-1 active glycoproteins from both brain and lymphoid sources and the most fully characterized of these is rat brain Thy-1.1 which has been sequenced with regard to amino acid composition (Willaims et al., 1981). Sialic acid containing glycoproteins and gangliosides have been isolated and exhibit Thy-1.1 or 1.2 specificity when assayed by immune response to Thy-1 in an allogeneic system (Wang et al., 1978, Kato et al. 1979, Wang et al. 1980). The expression of Thy-1 antigenic determinants may be dependent on the carbohydrate portion of these glycoconjugates although this has not been established. Observations on the glycoconjugate nature of Thy-1 led us to study the sialic acid portion of Thy-1 glycoprotein by radiolabeling cell surface sialic acid by periodate/borohydride treatment.

Introduction of tritium onto the sialic acid moieties of Thy-1 glycoprotein was accomplished by mild oxidation with periodate followed by reduction with tritiated sodium borohydride. This treatment has been used to label sialoglycoproteins of normal lymphoid cells and tumor cells (Lenten & Ahswell, 1971; Standring et al., 1978). The reaction is based on the susceptibility of C_8 and C_9 cis

hydroxyl groups of sialic acid to periodate oxidation. The periodate does not penetrate the membrane at 4°C thereby allowing only the oxidation of surface molecules. Using mild conditions, it has been shown that this method causes very little oxidation of other membrane proteins, lipids or carbohydrates (Gahmberg & Anderson, 1977). We have shown that treatment of periodate/borohydride labeled Thy-1 glycoprotein with mild acid or neuraminidase (which both remove sialic acid) resulted in almost complete removal of incorporated radioactivity (Figure 2 & 3). Thus periodate oxidation and sodium borohydride reduction has been shown to be a convenient and specific method for labeling cell surface Thy-1 in terminal sialic acid moieties. Further most of the properties of sialic acid remain unchanged because of the intact carboxyl group. For example, this treatment had no affect on the binding of antibodies to Thy-1, nor did it affect the immunoprecipitation of Thy-1.

Both xenogeneic and allogeneic monoclonal anti-Thy-1 antibody were used in immunoprecipitation experiments. Highly efficient precipitation was observed with xenogeneic monoclonal antiserum and only a glycoprotein of approximately 29,000 Da from Thy-1.2 thymocytes was precipitated (Figure 1). This demonstrates that Thy-1 glycoprotein was labeled with periodate/borohydride treatment and that the antigenicity was not altered by the labeling process. Allogeneic monoclonal anti-Thy-1 antisera

were also used and a glycoprotein of the same apparent Mr was also precipitated (Figure 1). The yield in radioactivity precipitated was about 20% of that obtained by the xenoantibody because the alloantibodies have been found to precipitate a distinct glycoprotein as discussed below.

The radiolabeled Thy-1 glycoprotein had an apparent Mr of 29,000 Da and appeared as one of major radiolabeled glycoproteins of mouse thymocytes. Three other major radiolabeled glycoproteins observed have approximate Mr of 100,000, 45,000 and less than 20,000 Da. Rat thymocytes labeled with periodate/borohydride had similar SDS-gel electrophoretic patterns as that of mouse thymocytes; including both a 25 to 29,000 Da and a 100,000 Da glycoprotein (Standring, et al, 1978). The 100,000 Da glycoprotien may be the Lgp 100 previously reported (Ledbetter et al., 1979; Durda et al., 1979). Variable amounts of radiolabeled material was usually observed at the dye front which was probably labeled glycolipids and lipids. The radioactivity of this very low molecular weight material was largely eliminated by ethanol precipitation of the glycoproteins prior to immunoprocipitation.

The four major radiolabeled species were all sensitive to neuraminidase hydrolysis but the sialic acid residues on 100,000 Da glycoprotein were hydrolyzed by neuraminidase at a slightly faster rate than those of Thy-1 glycoproteins and other radiolabeled glycoproteins (Figure 3). For example

the radioactivity of 100,000 Da glycoproteins decreased by 75%, after 4 hours of neuraminidase treatment whereas, the radioactivity of 50,000, 29,000, 20,000 Da glycoproteins decreased only by 40%, 50%, 55% respectively. This indicated that there may be differential susceptibility to neuraminidase treatment of the 100,000 Da glycoprotein possibly caused by conformation or linkage of the carbohydrate. The glycoprotein of intact cells treated with Nase and then radiolabeled were all available to the enzyme. Again the 100,000 Da glycoprotein appeared to be slightly more sensitive because it decreased 84% compared to approximately a 50% decrease observed in other labeled glycoproteins.

Nonequilibrium pH gradient electrophoresis of periodate/borohydride labeled and immunoprecipitated Thy-1.2 exhibited broad pI heterogeneity. The individual components observed were compared to those obtained by immunoprecipitation of iodinated Thy-1.2 glycoprotein (Figure 4). The proportion of radioactive sialic acid to polypeptide radioactivity (^{125}I) present in each component increased from the most basic to the most acidic form. This labeling pattern supports the notion that pI heterogeneity was contributed by sialic acid. Further, when these samples were treated with Nase and then analyzed by NEPHGE there was a loss of tritium accompanied by a shift toward a more basic pH (Figure 5). After 60 minutes of Nase treatment the Thy-1

components (indicated either by ^{3}H or ^{125}I radioactivity) shifted to more basic fractions. Further Nase treatment resulted in a greater loss of radioactivity associated with sialic acid. The Nase product of iodinated Thy-1.2 also shifted to one peak without loss of ^{125}I radioactivity. Continued Nase treatment of this peak did not result in further cathodal shift suggesting that the removal of the last sialic acids did not greatly shift the pI of the molecule. This is in contrast to the regular (3 fraction) shift observed upon addition of sialic in the more acidic components as seen in Figure 5.

Studies of the pI heterogeneity of Thy-1 glycoprotein have indicated that there are about five distinct components which are precipitable with xenogeneic anti-Thy-1.2 antibodies. Further Nase treatment results in a cathodal shift to more basic components (Hoessli et al., 1980; Ledbetter & Herzenberg, 1979; Wang et al., 1982). We have previously found that there are two distinct families of molecules which are recognized by anti-Thy-1.2 antibodies (Wang et al., 1982). The more acidic family which accounts for 80 to 90% of the thymocyte Thy-1.2 was precipitated from NP-40 lysates with xenogeneic anti-Thy-1.2 both before and after Nase treatment. The second family accouting for only 10 to 20% of thymocyte Thy-1.2 was precipitated by allogeneic anti-Thy-1.2 antibodies. This finding accounts for the fact that the xenogeneic antibodies precipitated

more radioactivity than the allogeneic antibodies as shown in Figure 1. Further the efficiency of precipitation of the minor allogeneic recognized component was decreased after Nase treatment thus accounting from the decrease in Thy-1 activity after Nase treatment described in previous reports (Wang et al., 1982, Wang et al., 1980).

Periodate/borohydride labeling of sialic acid revealed that the predominantly labeled family of Thy-1.2 was recognized by the xenogeneic anti-Thy-1.2 (Figure 4). This antibody precipitated a substantial amount of labeled Thy-1.2 from a thymocyte lysate. The family of heterogeneous pI corresponded exactly with those precipitated from surface iodinated cells (Figure 4). The small amount of precipitate of periodate/borohydride labeled Thy-1 observed with allogeneic anti-Thy-1.2 was considered to be due to the small amount of the allogeneic recognized form of Thy-1.2 in the NP-40 thymocyte membrane lysate; and to the apparent weak labeling with periodate/borohydride of the allogeneic form of Thy-1.2 compared with the predominant xenogeneic form. The latter observation was probably due to a lessor amount of sialic acid on this component which is suggested by its more basic nature compared to the xenogeneic recognized form which is more acidic and reacts well with periodate/borohydride.

In summary we have shown in this report that the sialic acid of Thy-1.2 glycoprotein is easily labeled with

periodate/tritiated borohydride treatment and the incorporated radioactivity correlates with the expected ratio of sialic acid to polypeptide. Neuraminidase treatment removed sialic acid and caused a cathodal shift of Thy-1.2 glycoprotein which compared with the shift observed with ^{125}I labeled Thy-1.2 glycoprotein. The predominantly labeled component was easily precipitated with xenogeneic anti-Thy-1.2 but only a small portion of the radioactivity could be precipitated with the allogeneic anti-Thy-1.2. These studies suggest that the role of Thy-1 on the T cell membrane as well as in neuronal tissue may involve sialic acid because this carbohydrate is present in relatively large amounts and provides a more acidic nature to the Thy-1.2 polypeptide which is possible the most basic molecule on the thymocyte membrane.

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APPENDIX

DETECTION OF THY-1.2 MEMBRANE COMPLEXES SHED FROM THYMOCYTES AND LYMPHOMA CELLS BY AN IMMUNORADIOMETRIC ASSAY

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Abstract—A direct binding immunoradiometric assay (IRA) for Thy-1 antigen was developed to study the properties of membranous complexes shed from murine thymocytes and lymphoma cell lines. Monoclonal anti-Thy-1.2 antiserum was iodinated and used to study the shedding from AKR (Thy-1.1) and C3H(Thy-1.2) thymocytes, and S49.1(Thy-1.2), S49-Thy-1$^-$ and BW5147(Thy-1.1) continuous lymphoma cell lines. Culture supernatant fluids or purified shed complexes were allowed to bind to microtiter plates followed by measurement of the binding of iodinated anti-Thy-1.2. The assay was found to be completely specific for the Thy-1.2 allotype, and in conjunction with antibody coated wells could detect Thy-1 solubilized from cells with N-P40 detergent. Shed complexes containing Thy-1 from thymocytes and lymphoma cell lines were analysed by isopycnic centrifugation with continuous potassium tartrate gradients (5–40%). Shed complexes had a buoyant density of 1.06–1.10 g/cm^3 as compared to 1.15–1.17 g/cm^3 expected for murine leukemia virus or 1.20–1.24 g/cm^3 expected for mycoplasma. We concluded that the shed membranous complexes had a buoyant density similar to plasma membrane and the complexes were similar from both thymocytes and lymphoma cell lines. Thy-1 was not associated with virus, mycoplasma or other particles found in the gradients and Thy-1 was not found in the unsedimented fraction. The release of Thy-1 from thymocytes and cultured cell lines results in only one defined density of particles which may participate in cellular communication or in the survival of malignant cells.

INTRODUCTION

The shedding of membrane components and fragments from normal and neoplastic cells is believed to occur in a wide variety of cases (Doljanski & Kapeller, 1976). The appearance of tumor antigens in the serum of tumor bearing hosts has been well documented and has been proposed as a mechanism by which tumors avoid immunological destruction (Alexander, 1974). Large membranous complexes or vesicles are of particular interest because they may carry a variety of antigens and receptors which may serve in cellular communication. Shed membrane complexes of high molecular weight have been identified which were taken up by other cells at distant sites (Cone *et al.*, 1971). Large membrane vesicles (enriched in membrane antigens and enzymes) appeared in the serum and in the ascites fluid of mice bearing leukemia in an ascites form (Raz *et al.*, 1978). These vesicles have been proposed to be involved in modulating the host response to the tumor cells. The vesicles have a high molar ratio of cholesterol to phospholipid and have a greater microviscosity than the leukemia cell membranes. The molecular nature of large shed complexes is not well understood but proposals have been forwarded that they arise from the 'pinching off' of microvilli or membrane 'blebs' (Koch & Smith, 1978).

The molecular and immunological nature of shed membrane complexes has been a subject of investigation in our laboratory. Membrane complexes have been isolated by column chromatography from the culture media of lymphoma cell lines as well as from the supernatant fluid of activated T cells (Freimuth *et al.*, 1978, 1979; Esselman & Miller, 1977). These complexes were found to be immunomodulatory and immunosuppressive in *in vitro* immune function assays, and they contained many membrane components including the Thy-1 antigen. Characterization of the nature of shed Thy-1 antigen was accomplished using the *in vitro* plaque forming cell assay which was specific for Thy-1 (Freimuth *et al.*, 1978, 1979).

This paper describes the development of a immunoradiometric assay for shed membrane complexes containing Thy-1 antigen which uses monoclonal anti-Thy-1.2 antibody. The assay was found to be effective for studying

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large numbers of samples derived from fractionation procedures used in the characterization of membrane complexes.

METHODS

Cell lines and culture methods

Murine lymphoma cell lines [BW5147-(Thy-1.1), S49.1(Thy-1.2), S49(Thy-1$^-$) and K36.3(Thy-1-)] were obtained from the Salk Cell Culture and Distribution Center, La Jolla, CA. These cell lines have been previously described in detail (Hyman & Trowbridge, 1977) and are of interest because of their variations in expression of Thy-1 antigen. They were all maintained in Dulbecco's modified Eagles medium (D-MEM*) (Grand Island Biological Co., Grand Island, NY) with 10% heat inactivated fetal calf serum (KC Biological Inc., Lenexa, KS) supplemented with dextrose (4.5 g/l) and $NaHCO_3$(0.6 g/l). No antibiotics were used. Cells were grown to a concentration of 1–2 × 10^6 cell/ml before passage. Viabilities were greater than 95% as determined by trypan blue exclusion and the cells had a doubling time of about 20 hr.

All of the cultures used were routinely tested for the presence of mycoplasma by electron microscopy. Fluorescent benzimidole staining (Russell *et al.*, 1975) and by [^{3}H]thymidine incorporated into 'cytoplasmic' DNA (Snyder & Stanbridge, 1975). A positive control for these assays consisted of cell cultures inoculated with *Mycoplasma hyorhinos* obtained from the Michigan Department of public Health. This strain of *Mycoplasma* has previously been identified as a contaminant of lymphoma cell lines (Wise *et al.*, 1978).

Direct binding immunoradiometric assay

Flexible, disposable 'V' well microtiter plates (Cooke Engineering Co., Alexandria, VA) were etched by adding and immediately removing toluene from the wells. The flexible plates were placed into a rigid microtiter plate for support during sample handling. A test sample of up to 150 μl was added to the wells and incubated at room temperature overnight. The test samples were removed and the wells were washed twice with PBS, and filled with 10% BSA in PBS, pH 7.5. The 10% BSA solution was removed after 5 min and the wells were washed once with PBS. One hundred microliters of the iodinated anti-Thy-1.2 sera diluted to 500 Kcpm 0.1 ml in 5% BSA were incubated in the wells for 1 hr at room temperature. The wells were washed five times with PBS to remove any unbound label. For antibody coating of the wells 150 μl of anti-Thy-1.2 (2 μg/ml in PBS, pH 7.2, 0.02% sodium azide) was added to each well. After 1 hr at room temperature the antisera was removed and saved. The wells were washed twice with PBS and the test sample was added as described above. Individual wells were freed for counting by slicing off the top of the microtiter plate with a hot 26 ga Nichrome wire heated with a 15 V transformer. The individual wells were placed in counting vials and counted for 1 min with a Beckman Bio gamma II gamma counter (Beckman Instruments, Fullerton, CA).

Antisera

Monoclonal anti-Thy-1.2 (Lot No. LK-185, New England Nuclear, Boston, MA) was tested for cytotoxic activity by trypan blue exclusion with C3H.HeJ and AKR.J thymocytes and bone marrow cells; and S49.1 and BW5147 lymphoma cells. Ten microliters of antisera were serially diluted with Dulbecco's MEM into tubes followed by the addition of 10 μl of cells at a concentration of 2.5 × 10^7 cells/ml. Ten microliters of adsorbed guinea pig serum (complement) diluted 1:3 with D-MEM were added and the tubes were incubated at 37°C. After 1 hr, the tubes were transferred to an ice bath and diluted with 50 μl D-MEM. Ten microliters of 0.4% trypan blue were added approximately 1 min before counting the cells. The monoclonal anti-Thy-1.2 sera used was found to be highly specific for Thy-1.2 and of high titer. The antisera was highly specific for Thy-1.2 and of high titer. The antisera was cytotoxic to S49.1 cells and C3H thymocytes to a titer of 1:61,440 (final dilution of antisera necessary to kill 50% of the cells). The antisera was not cytotoxic to AKR-thymocytes, C3H bone marrow cells or to BW5147 cells even at a dilution of 1:30. The labeled antisera also reacted only with Thy-1.2 shed complexes in the direct binding IRA but not to shed material from S49.1(Thy-1$^-$), BW5147(Thy-1.1) and K36.3(Thy-1$^-$) cells.

* Abbreviations used: IRA, immunoradiometric; PFC, plaque forming cell; D-MEM, Dulbecco's modified Eagles medium; BSA, bovine serum albumin; PBS, phosphate buffered saline.

Iodination

Monoclonal anti-Thy-1.2 was iodinated with $Na^{125}I$ (Amersham Searle, Arlington Heights, IL) by using the chloramine-T method (Greenwood *et al.*, 1963). Twenty micrograms of antiserum in 200 μl of PBS, pH 7.2, was mixed with 10 μl $Na^{125}I$ (100 μCi/μl) and 25 ng chloramine-T. The reaction was allowed to proceed for 15 min at 4°C and terminated by the addition of 50 ng sodium metabisulfite. Ten microliters of a 5% hemoglobin (type III, Sigma) 1 μ*M* NaI solution was added before separation of the unbound ^{125}I on a Sephadex G-50 column. The column was prepared by equilibration with 5% BSA and then washed with PBS, pH 7.2. The fractions containing the hemoglobin were then pooled and used in these experiments. Addition of hemoglobin after iodination of antisera served as an indicator of column fractions containing the labeled antibody and as a stabilizer protecting against denaturation.

Shed membrane complexes

C3H and AKR thymocyte shed material was prepared by incubating a suspension of thymocytes at a concentration of 10^8 cells/ml for up to 5 hr at 37°C in D-MEM. Cells were removed by pelleting at 4000 *g* for 15 min. Sodium azide (0.02%) was added to the supernatant which was stored at 4°C. Shed material from lymphoma cells S49.1, S49(Thy-1$^-$), BW5147 and K36.3 was obtained by incubating cells (2×10^7 cells/ml in D-MEM) for 2 hr at 37°C. The supernatant was prepared and handled as described above. A 1% N-P40 lysate was prepared by incubating 10^7 cells/ml 1% N-P40 in PBS, pH 7.2, for 1 hr at 4°C followed by centrifugation at 600 *g* for 20 min.

Potassium tartrate gradient

Shed membrane complexes were isolated from murine lymphoma cells and thymocytes by isopycnic sedimentation on linear gradients of potassium tartrate (Zwerner *et al.*, 1978). Two milliliters of the shed material was applied to a 13 ml linear gradient of 5–40% (w/w) potassium tartrate using a Beckman SW27.1 rotor in a Beckman L5-65 ultracentrifuge. The samples were centrifuged at 100,000 *g* for 90 min at 4°C. Gradients were fractionated into 0.5 ml volumes and densities were determined by refractometry (Abbe Refractionate, Fisher Scientific Co.).

RESULTS

A direct binding IRA for Thy-1 was devised by first studying the kinetics of the binding of shed membrane complexes containing Thy-1 to plastic culture wells. This was performed by binding C3H (Thy-1.2) thymocyte shed complexes to the wells of microtiter plates, blocking excess binding sites with 10% BSA and then assessing the binding of ^{125}I-anti-Thy-1.2 to the complexes. Increasing the initial sample incubation time produced a rapid and steady increase in binding of shed complexes as assessed by anti-Thy-1.2 binding (Fig. 1). The increase was greatest in the first 24 hr but steadily increased throughout a 70 hr incuba-

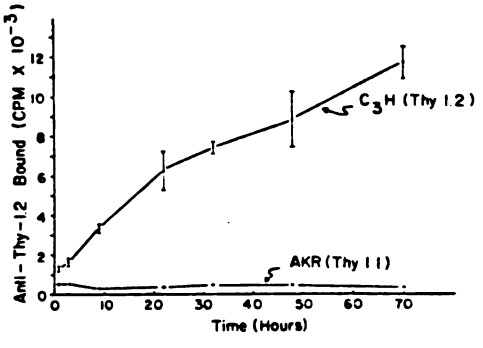

Fig 1. IRA of Thy-1 antigen shed from thymocytes. Membrane complexes shed from C3H(Thy-1.2) and AKR(Thy-1.1) thymocytes were bound to etched microtiter plate wells by incubation at room temperature. Excess antigen was removed at various times and [^{125}I]anti-Thy-1.2 binding was determined. The values shown are the mean ± S.E. of triplicate samples.

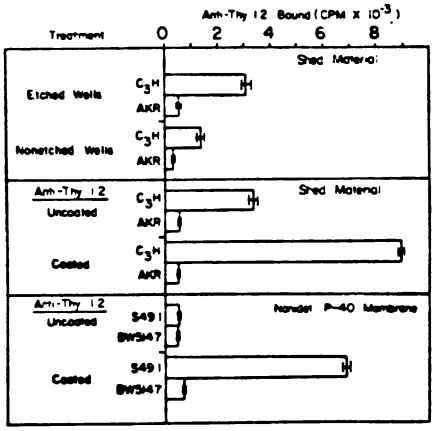

Fig. 2. Conditions for binding of shed complexes to microtiter plate wells. A comparison was made between binding of Thy-1.2 and Thy-1.1 shed material from thymocytes to toluene etched or antibody coated wells. Nonidet P-40 solubilized membrane from Thy-1.2 and Thy-1.1 lymphoma cells were also tested in antibody coated and uncoated wells. The values shown are the mean ± S.E. of triplicate samples.

tion without leveling off. During this period there was no change in the Thy-1.2 specificity because binding to shed complexes from AKR thymocytes remained at background levels. Although increasing the sample incubation time up to 70 hr increased the sensitivity of the assay, the 24 hr incubation used in these studies was found to be adequate. A similar study showed that the time required to block excess available sites on the wells with BSA was essentially complete after about 1 min of incubation, and the time required for binding ^{125}I-anti-Thy-1.2 was essentially complete after 1 hr of incubation (data not shown).

The sensitivity of this assay could be increased by adsorption of anti-Thy-1.2 antibody to the wells before antigen binding or by etching the wells with toluene. Etching the wells alone doubled the number of counts obtained from C3H shed complexes without significantly increasing the background (Fig. 2). Coating of the etched wells with anti-Thy-1.2 antibody prior to sample application again doubled the number of counts obtained from C3H shed complexes without an increase in the binding to AKR shed complexes. Besides increasing the sensitivity of the assay, anti-Thy-1.2 coating of wells enabled the detection of Thy-1.2 in the presence of 1% N-P40. Thy-1.2 solubilized from the surface of S49.1 cells by 1% N-P40 would not adhere to the wells even if the sample was extensively dialysed for 48 hr with PBS (Fig. 2). These results show that the direct binding IRA is specific for Thy-1.2 obtained from shed material or solubilized from lymphoma cell membrane with detergent.

The specificity of the assay and the anti-Thy-1.2 sera was further established by assay of the shed material from Thy-1.2 and 1.1 cell lines and their clones (Fig. 3). Only the cell line expressing Thy-1.2 (S49.1) gave positive results and the Thy-1$^-$ clone derived from this line was negative. Shed material from BW5147(Thy-1.1) and an AKR Thy-1$^-$ thymoma (K36.3) were both negative.

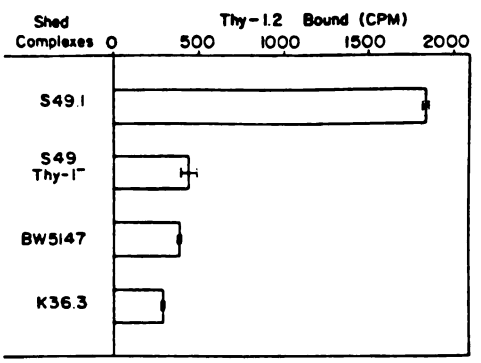

Fig. 3. Thy-1 activity of shed membrane complexes from lymphoma cell lines. The lines used were S49.1(Thy-1.2), BW5147(Thy-1.1), S49.1(Thy-1.2$^-$) and K36.3(Thy-1.1$^-$). The shed material was incubated in anti-Thy-1.2 coated, etched wells overnight and assayed with [^{125}I]anti-Thy-1.2. The values shown are the mean ± S.E. of three assays.

Culture supernatants derived from either thymocytes or lymphoma cells were fractionated by continuous density gradient centrifugation to detect the presence of Thy-1 containing membranous particles. Gradients were fractionated and all the fractions were assayed for the presence of Thy-1 by direct binding IRA. AKR and C3H thymocyte culture supernatant fluids were subjected to gradient analysis and the results indicated the presence of one major class of Thy-1.2 containing particles at very low density, 1.06–1.10 g/cm^3 (Fig. 4A). No binding activity was observed for AKR thymocytes, thus demonstrating Thy-1 specificity. The density obtained was somewhat lower than that expected for isolated membrane (1.12–1.13 g/cm^3), virus (1.15–1.17 g/cm^3) or mycoplasma (1.20–1.24 g/cm^3) (Wise *et al.*, 1978). The Thy-1 active particles clearly sedimented into the gradient, as indicated by the density determinations shown in Fig. 4(B), and no activity was observed in the unsedimented layer at the top of the gradient (Fractions 1–5). Analysis of lymphoma culture supernatants using the same technique gave similar results (Fig. 4B). A single sharp peak of Thy-1.2 activity was observed for S49.1 cells (1.06–1.10 g/cm^3) and no activity was observed for Thy-1 negative clone (S49 Thy-1$^-$). Thy-1 activity was not observed above the gradient or lower in the gradient where virus or mycoplasma sediment. These results indicate that the lymphoma cells are releasing into the medium a single species of Thy-1 containing material of very low density.

The presence of mycoplasma in culture supernatants must be rigorously eliminated in a study of shed complexes. We have performed

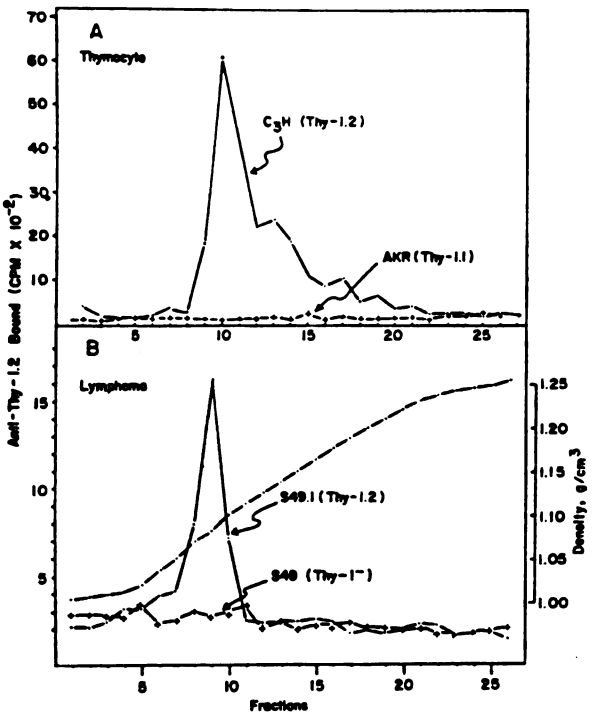

Fig. 4. Isopycnic centrifugation of shed material on linear potassium tartrate gradients. Linear gradients of potassium tartrate were formed and shed material was centrifuged at 100,000 *g* for 90 min at 4°C. (A) Analysis of 2 ml of supernatant from C3H and AKR thymocytes incubated 3 hr in D-MEM without FCS at a concentration of 1 × 10^8 cells/ml. (B) Analysis of 2 ml of supernatant from S49.1 or S49(Thy-1$^-$) lymphoma cells incubated for 2 hr in D-MEM without FCS at a concentration of 2 × 10^7 cells/ml. Fractions were assayed for Thy-1 activity and for density as described in Methods.

various assays to test for mycoplasma. As positive controls, cell lines were infected with *Mycoplasma hyorhinis*. Cell lines were compared with infected cell lines by three different methods (see Methods), all of which demonstrated no mycoplasma contamination. For example, when [^{3}H]thymidine incorporation into 'cytoplasm' was compared to nuclear incorporation for mycoplasma infected cells, a level of 30% incorporation into 'cytoplasm' was observed compared to 2–4% for uninfected cell lines. To eliminate further the possibility of latent contamination with mycoplasma (or other bacteria) all of the work described herein was performed with cultures free of antibiotics.

DISCUSSION

Shedding of both large membrane complexes and small molecules from cells has been proposed to function both in normal cellular communication and in the enhancement of survival of tumor cells. Our studies have concentrated on large membrane complexes released from viable cells because these carry a number of specific cell surface tumor antigens and normal antigens. Thy-1 antigen is an ideal focus for this study because it is a glycoconjugate which is expressed on the surface of T cells (and neuronal cells) and it has been well characterized. Murine Thy-1 exists in the membrane as a glycoprotein of about 24,000 daltons (Zwerner *et al.*, 1977; Letarte & Meghji, 1978) as well as a glycolipid containing sialic acid (Wang *et al.*, 1978; Kato *et al.*, 1979).

The release of Thy-1 from normal and lymphoma cells has been reported (Freimuth *et al.*, 1978, 1979), but the nature of the membrane complexes has not been fully determined. The object of the present study was to develop a reliable assay for Thy-1 which would be effective under various conditions used in the isolation and characterization of shed complexes. An immunoradiometric assay was chosen because other assays used previously (cytotoxicity and the immune response assay) were not sensitive or were easily disrupted by contamination with small amounts of the chemicals used in the isolation. The IRA was found to be suitable for assaying large numbers of samples derived by column or density gradient fractionation and allowed the determination of Thy-1 specificity in each assay, which was considered essential for the interpretation of our results.

A direct binding IRA was developed because of its simplicity compared to other techniques. The binding of Thy-1 containing shed complexes to untreated microtiter plate wells proceeded in a steadily increasing manner but did not reach completion in 70 hr under the conditions used. This was probably due to the dilute nature of the shed material because concentrated protein solutions (e.g. 1% BSA) quickly saturated the plate. The binding of anti-Thy-1 to complexes bound to the plate reached saturation within 1 hr of incubation. The conditions chosen for routine assays involved an overnight binding of shed material to the plates followed by a 1 hr incubation with iodinated anti-Thy-1.2 antibody. The sensitivity of the assay could be increased by lengthening the initial binding time or the concentration of the shed material. The binding to Thy-1.1 (BW5147 or AKR) shed material did not increase with these manipulations or with any of the other assays performed. Toluene etching of wells consistently increased adsorption of protein to all batches of plastic tested. Etching produced visible pitting of the wells, possibly increasing the area for adsorption or exposing new adsorption sites.

The advantages of using antibody coated wells included the increase in specific labeling as well as a reduction in background and sample variation. The antisera concentration used to coat the wells, 2 μg ml. could be reduced without decreasing specific binding. Denaturation of antibody stored at low concentrations was the major reason for using a higher concentration. We stored antibody coated wells for several weeks at 4°C with minimal deterioration. The use of an antisera to bind one antigen to the well and a different labeled antisera to detect another may be advantageous in determining the antigenic composition of complex shed material.

Esselman & Miller (1977) reported that Thy-1 containing shed material from antigen stimulated T cells nonspecifically modulated antibody responses. Freimuth *et al.* (1978), using a Sepharose-6B column to separate shed complexes from lymphoma cells, found that the majority of the Thy-1.2 associated complexes were primarily of high mol. wt (greater than 2×10^6 daltons). Further characterization of these complexes using density gradient centrifugation proved difficult because of the large number of fractions to be analysed by the immune response PFC assay and the necessity of maintaining sterile conditions. With the direct binding IRA reported here, identification of

fractions containing the Thy-1.2 shed complexes was rapid and sensitive.

The Thy-1.2 containing shed complexes were found to have a buoyant density of 1.06–1.10 g/cm^3 on potassium tartrate gradients. This was in agreement with our unpublished finding of a density between 1.04 and 1.13 g/cm^3 determined with a sucrose discontinuous gradient in which Thy-1.2 was detected with a PFC assay. The density of the shed complexes was somewhat lower than that expected for plasma membrane (1.12–1.13 g/cm^3). The differences are so small, however, that it will be necessary to compare the densities of plasma membrane and shed complexes on the same gradients. The greater spread of Thy-1.2 shed complexes from thymocytes (Fig. 4A) into more dense fractions may reflect the presence of plasma membrane fragments in the preparation. The density of the Thy-1 containing complexes indicate that the shed antigens were not bound to virus (1.15–1.17 g/cm^3) or to mycoplasma (1.20–1.24 g/cm^3), as had been previously suggested (Wise *et al.*, 1978). The possibility that Thy-1.2 antigen was carried by mycoplasma was further ruled out by fluorescent benzimidole staining, electron microscopy, and by comparison of the percentage total [^{3}H]thymidine incorporated into a 'cytoplasmic' DNA fraction. To increase the chances of detecting minimal mycoplasma contamination, all cultured cells used in these experiments were grown in media without antibiotics. Since the density of Thy-1.2 shed material from a primary culture of C_3H thymocytes was similar to that of continuously cultured cells, it seems even less likely that the shed complexes are composed of mycoplasma.

There is growing evidence that large membranous complexes are released by both transformed and normal cells. A number of tumor antigens have been described which are released from tumor cells into the surrounding medium and into the circulation of the host. Alexander (1974) has summarized the hypothesis of tumor escape from immune destruction through shedding of surface antigens. Membrane fragments similar to the Thy-1 containing complexes shed from lymphoma cells (Freimuth *et al.*, 1978; this report) have been reported by other investigators. Vitetta & Uhr (1972) demonstrated that normal splenic lymphocytes released cell surface immunoglobulins bound to membrane fragments devoid of H2 antigen. A similar selective release of Thy-1 (but not H2) was observed from thymocytes (Vitetta *et al.*, 1974). Calafat *et al.* (1976) observed liposome-like particles being released from leukemia cells in suspension culture. This shedding may have been due to 'pinching off' of microvilli, as was suggested by Koch & Smith (1978).

Inbar and associates have reviewed research concerning the characteristics of membranous vesicles isolated from ascites fluid of lymphoma bearing mice (Raz *et al.*, 1978). Membrane vesicles have been isolated from the ascites fluid or blood serum from mice bearing GR, EL_4 and YAC leukemia (Van Blitterswijk *et al.*, 1975; Raz *et al.*, 1978) and from the serum and pleural effusion of human acute lymphatic leukemia (Petitu *et al.*, 1978). The released vesicles have a high molar ratio of cholesterol to phospholipids and increased microviscosity compared to normal cells. The vesicles were also enriched for plasma membrane marker enzymes and lectin receptors. Vesicles isolated from Moloney virus-induced lymphoma bearing mice were highly enriched in plasma membrane marker enzymes and viral protein (P30) antigen (Raz *et al.*, 1978). The vesicles could be divided by differential centrifugation into virus free vesicles that induced immunity and fractions that induced tumors. Membrane vesicles have thus been found to be released from growing cells *in vivo*.

The complexes which we have reported herein have similar properties to vesicles described by Raz *et al.* (1978). They were collected from actively growing cells and are of a density somewhat lower than expected for plasma membrane. This is consistent with the proposal that they have a higher cholesterol to phospholipid ratio than plasma membrane. We have previously identified an immunomodulatory effect of culture media from activated T cells and lymphoma cells (Esselman & Miller, 1977; Freimuth, 1979). Preliminary results (unpublished) have indicated that all of the immunomodulatory activity of shed media was associated with the Thy-1 active complexes isolated from a tartrate gradient. Future studies will center on the elucidation of the potential effects that these complexes may have on host responses to lymphoma.

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RADIOLABELING AND ISOLATION OF THY-1 ACTIVE GLYCOLIPIDS FROM MURINE BRAIN AND LYMPHOMA CELL LINES[1]

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Murine Thy-1 active glycolipids were isolated and characterized by radiolabeling, thin layer chromatography (TLC), and an immune response-plaque forming cell (PFC) assay. Brain gangliosides were labeled *in vivo* by intracranial injection of [1-^{14}C]*N*-acetylmannosamine (ManNAc). Gangliosides were extracted, separated by one and two dimensional TLC, and visualized by autoradiography. The gangliosides isolated from AKR (Thy-1.1) and ICR (Thy-1.2) mice were assayed for Thy-1.1 and 1.2 activity with an anti-Thy-1 PFC assay. One Thy-1.1 active compound was identified out of about 15 AKR gangliosides. None of the AKR gangliosides had Thy-1.2 activity in the PFC assay. Similarly, one Thy-1.2 active ganglioside was identified out of the ICR gangliosides and none of these had Thy-1.1 activity. Thus the brain gangliosides demonstrated allogeneic specificity for Thy-1 antigen.

BW5147 (Thy-1.1) and S49.1 (Thy-1.2) murine lymphoma cell lines that express Thy-1 were also examined for Thy-1 active glycolipids. The cell lines were incubated *in vitro* with either [1-^{14}C]palmitate or [1-^{14}C]-ManNAc, and the glycolipids were extracted, separated by two dimensional TLC, and visualized by autoradiography. A Thy-1.2 active compound was identified from the S49.1 cells. None of the BW5147 glycolipids had Thy-1.2 activity and none of the S49.1 glycolipids had Thy-1.1 activity. Neuraminidase and mild HCl treatment of Thy-1 active glycolipids abrogated the subsequent PFC response. The Thy-1 glycolipids bound to DEAE cellulose as expected for gangliosides. We therefore conclude that Thy-1 antigenicity is associated with gangliosides of mouse brain and lymphoma cells.

The Thy-1 alloantigen is the product of co-dominant alleles on chromosome 9 in mice (1, 2). 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 that 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 (3). The Thy-1 glycolipid had properties similar to gangliosides and was isolated and partially purified with column chromatography and thin layer chromatography (TLC).[1] 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 (4). This assay demonstrated the allogeneic specificity of both glycoprotein and glycolipid forms of Thy-1.

Thy-1 antigenicity has been found to be present on a protein (5, 6) or glycoprotein molecule (7–9). The actual composition of the Thy-1 alloantigenic determinant has yet to be established for the glycoprotein. Barclay *et al.* (10) 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.* (8) established that a protein lipid interaction is necessary for Thy-1 antigenic activity. Trowbridge and Hyman (9) 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 biosynthetic 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 suggested by interactions with DEAE cellulose ion-exchange chromatography, and by neuraminidase treatment.

MATERIALS AND METHODS

Radiolabeling of brain glycolipids. Thy-1 glycolipid was labeled biosynthetically by using a previously described method (11). One litter of 7-day-old pups (5 to 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, Maine, and ICR Swiss mice were obtained from Spartan Research Animals, Inc., Haslett, Mich. Each mouse pup was injected intracranially with 8 μl of sterile saline solution containing 5 μCi [1-^{14}C]*N*-acetylmannosamine, 54.5 mCi/mmole (New England Nuclear, Boston, Mass.). This solution was injected into both sides of the head at a point 1 to 2 mm anterior to the intrauricular line and 2 to 3 mm lateral to the midline with a 10 μl Hamilton syringe (Hamilton Company Inc., Whitting, Calif.). The tip of the needle was introduced only far enough to completely submerge the bevel of the tip

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[3] Abbreviations: D-MEM, Dulbecco's modified Eagles medium with 10% fetal calf serum; Gal, galactosyl; Glc, glucosyl; GalNAc, *N*-acetylgalactosaminyl; ManNAc, *N*-acetylmannosaminyl; NeuAc, *N*-acetylneuraminyl; cer, 2-*N*-acylsphingosine; G_{M1}, Gal-GalNAc-Gal(NeuAc)-Glc-cer; G_{D1a}, Gal(NeuAc)-GalNAc-Gal(NeuAc)-Glc-cer; G_{D1c}, Gal(NeuAc-NeuAc)-Glc-cer; other gangliosides are identified according to reference 24.

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 were isolated as described below. Incorporation of *N*-acetylmannosamine (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 (La Jolla, Calif.). 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, N. Y.) containing 1000 mg/l glucose, with 10% heat inactivated fetal calf serum (FCS, Grand Island Biological Co.), and either 2.0 μCi/ml [1-^{14}C]*N*-acetylmannosamine, 54.5 mCi/mmole, or 2.0 μCi/ml [1-^{14}C]palmitic acid, 30 mCi/mmole (International Chemical and Nuclear Corp., Irvine, Calif.). Cells were cultured at 37°C in flasks at a concentration of 5×10^5 cells/ml. The cells reached a concentration of 2×10^6 cells/ml after 48 hr. 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 (12). The total lipid extract was subjected to a Folch partition (13), and the ganglioside rich upper phase was dried *in vacuo* and hydrolyzed with mild alkali by using 0.3 N NaOH in methanol-chloroform (1:1, v/v) for 30 min at room temperature. This mixture was neutralized with glacial acetic acid, evaporated, resuspended in distilled H_2O, and dialyzed for 48 hr at 4°C 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) mice were separated by column chromatography by using Anasil S (Analabs Inc., North Haven, Conn.) with chloroform-methanol-water (C:M:W) mixtures (14). Column fractions were pooled and prepared for Thy-1 testing by 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 with Silica Gel 60 TLC plates (E. Merck, Darmstadt, West Germany). Dialyzed upper phase samples of brain and lymphoma cells with between 30 to 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 with quantitative gas-liquid chromatography. The TLC-developed standards were visualized with I_2 vapors and served as a reference to comapre 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_{D1}) as described by Esselman *et al.* (12). 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_{M1} was isolated as well as the band directly above G_{M1}. 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 by 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 1 and once in the second dimension in solvent 2. 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 inch sheet of Kodak SB-5 X-ray film (Eastman Kodak Co., Rochester, N. Y.) and kept at 4°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 (No. 146-5327) and fixed in Kodak Rapid Fix (No. 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 1 hr at 37°C. The sample was heated to 100°C for 15 min, 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 five-column volumes of chloroform-methanol-ammonium acetate (12). 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 min (12). 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 (4) originally developed an *in vitro* PFC assay that detected Thy-1 alloantigen on whole thymocytes. A modified version, used here, has previously been described and found to be effective for measuring the immune response to isolated glycolipid and glycoprotein Thy-1 alloantigens (3).

The spleen cells for the anti-Thy-1.1 PFC assay were obtained from C3H/HeJ mice primed with a single i.v. injection of 4×10^7 AKR/J thymus cells in Eagle's minimal essential medium (MEM) 2 to 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 thymic cells in MEM. One milliliter 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, resus-

pended, and incubated in 0.5 ml CMRL 1066 containing 15% FCS for 1 hr 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 was determined. Target cells for the anti-Thy-1 PFC assay were obtained from the thymuses of 10- to 16-week-old AKR/J or C3H/HeJ. Cultured spleen cells were centrifuged; the pellets were resuspended in 0.1 ml MEM containing 2×10^7 viable 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, N. Y.) were maintained in a 50 to 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 for 5 to 5.5 hr at 37°C in a humid 8% CO_2 atmosphere upside down in plastic slide trays; D-MEM/FCS was added to cover each slide. Rabbit complement (C) (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 (15) in which slides were drained; 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; the dark trypan blue stained plaques were counted by using a dissecting microscope. The PFC response for each glycolipid antigen was an average of five cultures. Standard errors were calculated and the Student *t*-test was used 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.

Thy-1 glycoprotein. Glycoprotein extracted and highly purified from C57BL/6 mouse thymus (7) was a generous gift from Dr. M. Letarte (Toronto, Canada). *Clostridium perfringens* neuraminidase, 0.1 units, was added to the glycoprotein (1 μg) in acetate buffer, pH 4.5, and incubated for 1 hr at 37°C. The sample was heated to 100°C for 1 min to destroy residual enzyme activity and added to the PFC cultures to test for remaining Thy-1 activity.

RESULTS

Mouse brain glycolipids were separated by column chromatography with Anasil S by using previously published procedures as described in *Materials and Methods*. Glycolipid fractions were pooled and tested for Thy-1 antigenicity by using the anti-Thy-1 PFC assay. Thy-1 glycolipid was previously shown to be present only in pooled fractions containing G_{M1} ganglioside (3).

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 Fig. 1) were eluted from the silica gel, divided, and assayed for Thy-1.1 and 1.2 antigenicity by using the anti-Thy-1 PFC assays (Fig. 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 (see 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 (3). 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}.

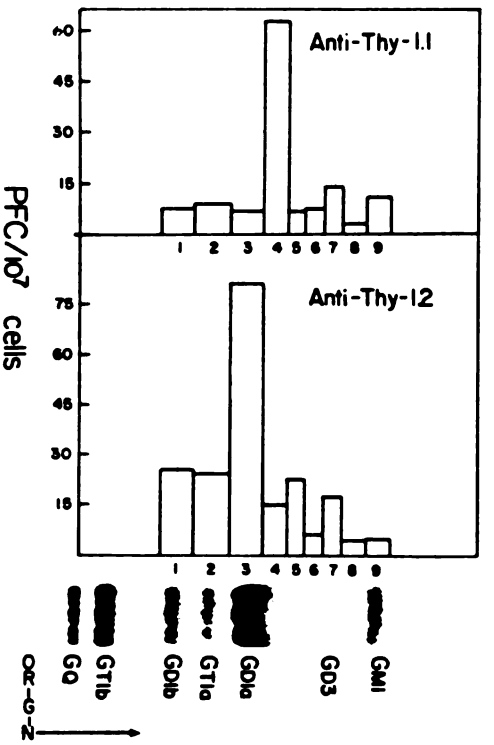

Figure 1. Thin layer chromatography of Thy-1. Column G_{M1} fractions containing Thy-1 glycolipid from AKR and C3H brain were mixed and chromatographed in solvent 1. The sample was eluted in fractions, divided, and assayed in the anti-Thy-1.1 and anti-Thy-1.2 PFC assays. Standard mouse brain gangliosides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

Thy-1 glycolipid could not be visualized on the TLC plate in Figure 1 by using I_2 vapors or spray reagents because of the minute quantities 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 (Fig. 2). Thy-1 glycolipid had a mobility in solvent 1 that was slightly greater than, but generally contaminated with, G_{D1a} ganglioside as shown in Figure 1. Thy-1 was not observed on the first plate (step 1, Fig. 2) because of this contamination. The area (A), corresponding to G_{D1a} ganglioside and slightly above (but not including G_{D3}), was eluted from the silica gel, applied to a second TLC plate, and developed in solvent 2 (step 2). Previous

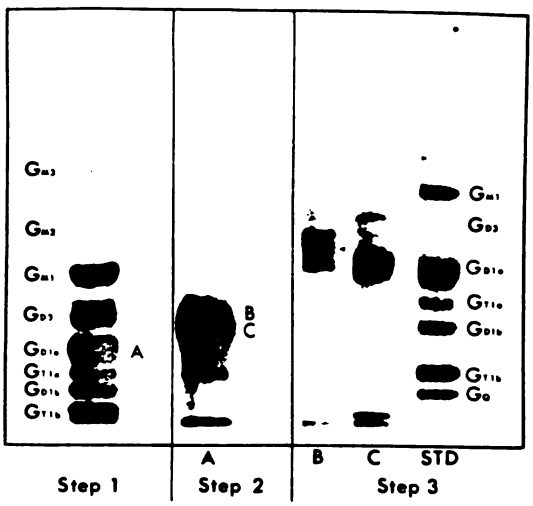

Figure 2. One dimensional TLC of radiolabeled Thy-1 glycolipid with sequential solvents. ICR brain ganglioside, radiolabeled with [1-^{14}C]ManNAc, were chromatographed in solvent 1 (step 1). The Thy-1 active area (*A*) was eluted and chromatographed in solvent 2 (step 2). The Thy-1 active area (*B*) 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.

work in our laboratory (3) indicated that Thy-1 had a mobility in solvent 2 that was slightly greater than G_{M1} and coincident with G_{D1}. 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 on one thin layer plate. AKR/J (Thy-1.1) or 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 (Fig. 3). These spots were identified by relative TLC mobility compared to the ganglioside standards on the side of the plates (not shown in Fig. 3). The AKR brain gangliosides were tested with the anti-Thy-1.1 PFC assay; the ICR brain gangliosides were tested with the anti-Thy-1.2 PFC (Fig. 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_{D1} and to the right of G_{D1a} in the orientation shown (Fig. 3). Brain Thy-1.1 glycolipid (fraction 4) consistently migrated slightly faster in solvents 1 and 2 than Thy-1.2 glycolipid (fraction 4). By using G_{D1} as a reference, Thy-1.1 was further to the right and below G_{D1} than Thy-1.2, which was directly below G_{D1} (Fig. 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 by using the extraction and two-dimensional TLC procedures mentioned above. A number of spots were revealed by autoradiography of [1-^{14}C]palmitic acid-labeled glycolipids (Fig. 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

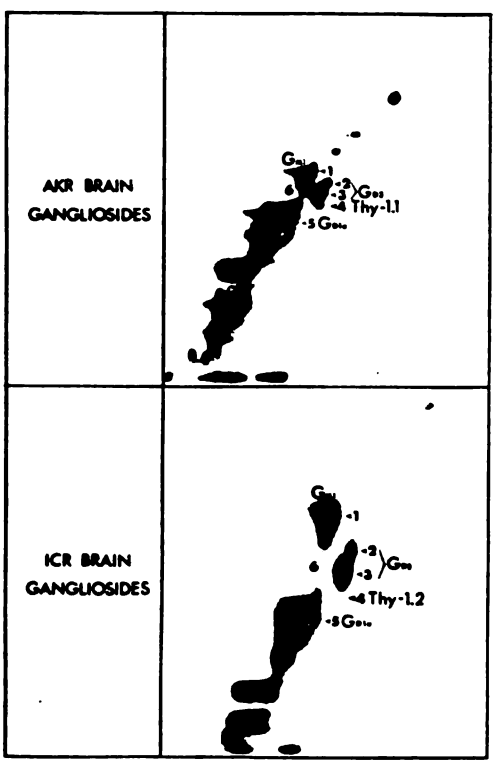

Figure 3. Autoradiograms of ManNAc labeled brain gangliosides. Gangliosides labeled by intracranial injection of [1-^{14}C]ManNAc were extracted from 1g of brain, applied in one spot on a TLC plate and were separated in two dimensions with solvent 1 (*vertically*) and solvent 2 (*horizontally*). The assayed fractions indicated by numbers (and ganglioside abbreviations) correspond to the PFC assay results in Figure 4. Fraction 6 refers to the area surrounding all the assayed spots.

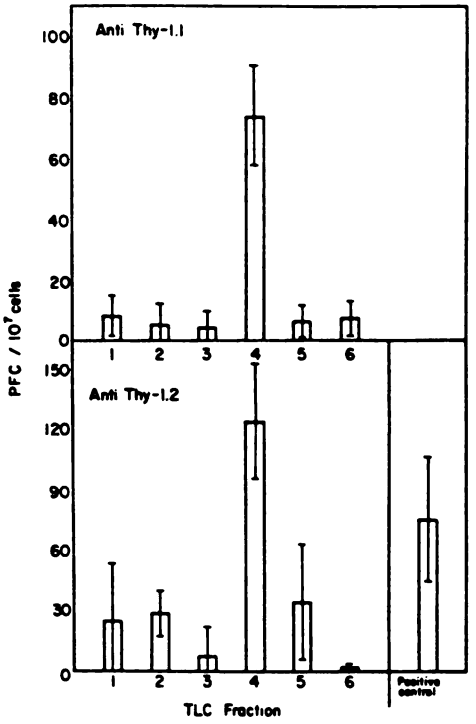

Figure 4. Anti-Thy-1 PFC assay of brain gangliosides. The gangliosides assayed were eluted from the plate shown in Figure 3, diluted and the amount derived from 0.1 g of brain was added to the PFC assay cultures. The values shown are the mean ± standard error of five cultures. Application of the Student *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.

PFC assay; 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, Fig. 6) and one Thy-1.2 glycolipid from S49.1 cells (fraction 7, Fig. 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 (Fig. 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 by using two-dimensional TLC from brain (AKR/J and ICR) and lymphoma cells (BW5147 and S49.1) were tested for 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 (Fig. 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. We have previously demonstrated the Thy-1 specificity of purified Thy-1 active glycoprotein by using the Thy-1 PFC assay (3).

DISCUSSION

We have previously reported on the occurrence of glycolipids from mouse brain and thymocytes that carry Thy-1 antigenicity and specificity (3). Other researchers have reported on the isolation of Thy-1 active glycoproteins from rat (7) and murine (16) lymphoma cells. The immune response assay we have used recognized both of these antigen types as well as demonstrating their allogeneic specificity (3). Precedent for similar carbohy-

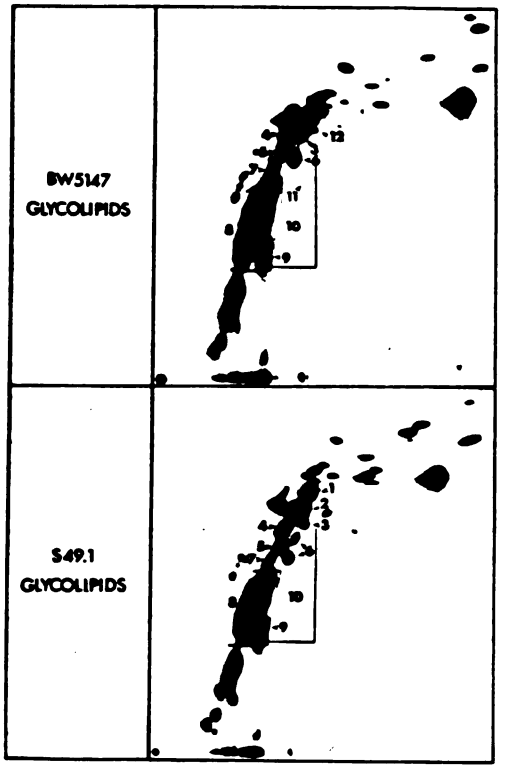

Figure 5. Autoradiograms of Palmitate-labeled lymphoma cell glycolipids. Chromatography was performed as described in Figure 3, with the gangliosides derived from 2 × 10⁸ cells. The assayed fractions are labeled with numbers that correspond to the anti-Thy-1 PFC assay in Figure 6. The number 10 refers to the area surrounding the spots that were assayed.

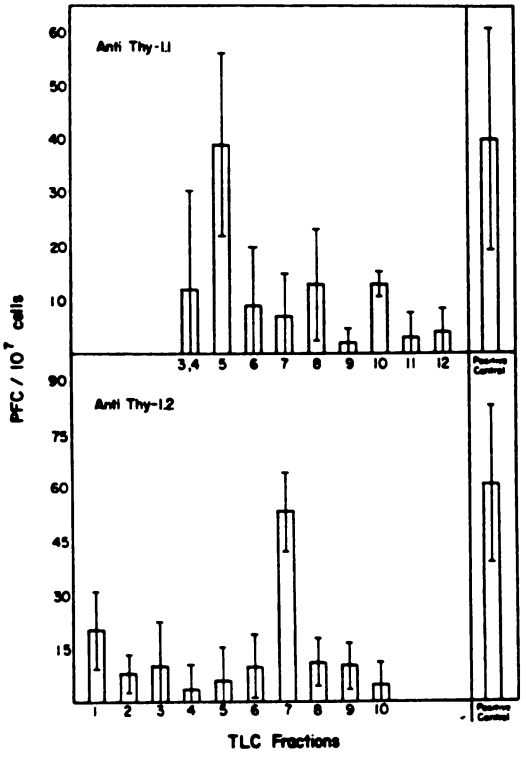

Figure 6. Anti-Thy-1 PFC assay of lymphoma glycolipids. Glycolipids were eluted from the TLC plate shown in Figure 5. The glycolipids derived from 2×10^7 cells were added to each culture. The values shown are the mean ± standard error of five cultures. Application of the Student *t*-test to the standard errors for these samples resulted in p values less than 0.05 when compared to the Thy-1 active fraction. Positive controls are brain Thy-1 glycolipids.

drate determinants on glycoprotein and glycolipid antigens is found in the ABO, Lewis blood group systems (17, 18) as well as in the report of Tonegawa and Hakomori (19) on the nature of "ganglioproteins"—that is those proteins that share antigenic moieties with gangliosides. 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 that is a precursor of sialic acid and has been previously shown by neuraminidase hydrolysis to be incorporated exclusively into the sialic acid moieties of gangliosides (11). [1-^{14}C]palmitic 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 (11, 20, 21). Phospholipids and triglycerides were eliminated from our preparations by mild base hydrolysis and dialysis (12).

Radiolabeled Thy-1 glycolipid was isolated from ICR mouse brain by using sequential solvents in one-dimensional TLC (Fig. 2). Autoradiography of these TLC plates identified a glycolipid band with Thy-1 activity (Fig. 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 by 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 and solvent 2. The mobility of Thy-1 glycolipids (Fig. 3) was very similar to G_{D1} ganglioside in that they migrated ahead of G_{M1} in solvent 2, and behind G_{M3} and slightly ahead of G_{D1a} in solvent 1. We have also found that Thy-1.1 migrates slightly

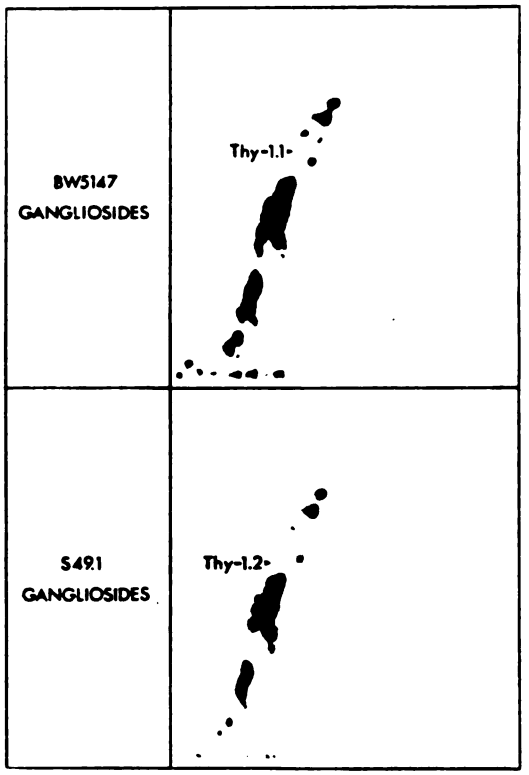

Figure 7. Autoradiogram of ManNAc-labeled lymphoma cells. Lymphoma cells were labeled with [1-^{14}C]ManNAc in culture. Chromatography was performed as in Figure 3 with the extract of 2×10^7 cells. Thy-1 glycolipids are indicated in this figure.

TABLE I

Properties of Thy-1 active glycoconjugates

Glycoconjugate	Treatment	Thy-1.1 (Anti-Thy-1.1 Response)	Thy-1.2 (Anti-Thy-1.2 Response)
Glycolipid[a]	None	51 ± 8	63 ± 6
	C.p. Nase (1 hr)[b]	13 ± 6	15 ± 9
	HCl[c]	1 ± 1	7 ± 2
	DEAE: bound[d]	36 ± 5	45 ± 9
	DEAE: not bound	8 ± 6	1 ± 1
Glycoprotein[e]	None	ND	58 ± 16
	C.p. Nase (1 hr)	ND	29 ± 16
	C.p. Nase (24 hr)	ND[f]	7 ± 5

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

[b] *Clostridium perfringens* neuraminidase was incubated with glycolipids for 1 hr or 24 hr at 37°C.

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

[d] DEAE chromatography was performed as described in the text.

[e] 100 ng of purified Thy-1.2 glycoprotein was used for these experiments.

[f] Not done.

faster than Thy-1.2 in solvent system 1 (Fig. 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 that 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 (Figs. 5 and 7). Additional compounds labeled with palmitate (Fig. 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 were resistant to mild base treatment that 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 1 hr 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 (Fig. 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; the ICR strain and the S49.1 cell line were positive in the anti-Thy-1.2 PFC assay. No cross-reaction was observed at the levels tested. Demonstration of the reciprocal allogeneic specificity is important because it supports the suggestion that the glycolipids carry specificities that parallel the serologic 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 (22).

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; 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 immunologic specificities.

For lymphoma cells, glycolipids extracted from 2×10^7 cells were added to each culture; for brain the gangliosides extracted from about 10 mg were used. When greater amounts of antigen were added, greater activity was observed. The PFC assay we used was extremely sensitive and gave consistent responses over a broad range of antigen amounts. Under the conditions of the assay, no other glycolipids were observed to have Thy-1 activity other than those described herein. This does not however preclude the possibility that other glycolipids might exhibit cross-reactivity if tested in different assays. Many other immunologic assays for Thy-1 however are ineffective for glycolipids because of the low binding usually observed between these compounds and antibodies. Assays for Thy-1 have been difficult to use or have given very low levels of binding with the Thy-1 glycoprotein. Letarte (23) has observed only weak interactions of Thy-1 glycoprotein with antibody in radioimmunoassays and absorption assays and has proposed that this may be due to the low affinity of mouse anti-Thy-1 sera.

In summary, identification of the minute quantities of Thy-1 glycolipids in brain and lymphoma cells was possible by incor-

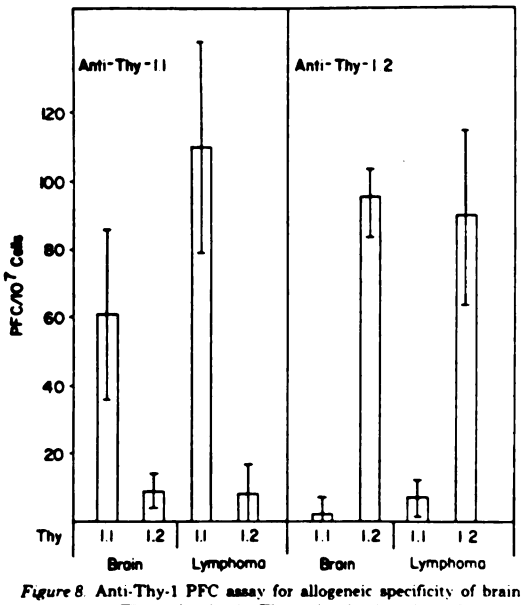

Figure 8. Anti-Thy-1 PFC assay for allogeneic specificity of brain and lymphoma Thy-1 glycolipids. Thy-1 glycolipids isolated from the two dimensional TLC plates in Figures 3 and 5 were tested for allogeneic specificity with the anti-Thy-1 PFC assays. The values shown are the mean ± standard error of five cultures. Application of the Student *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.

poration 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 serologic 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. The exact nature of the Thy-1 antigenic determinant is not yet fully known. It is quite possible that the "Thy-1 active" determinants on glycoproteins and glycolipids have different structures. We have shown, however, that the thymocytolytic plaque assay is specific for Thy-1 antigens and the glycoprotein and glycolipids both exhibit similar Thy-1 activity (3). Furthermore, the activity of both the glycolipid and the glycoprotein was destroyed by neuraminidase treatment (Table I) confirming a previous report by Johnson *et al.* (25). Thus the Thy-1 active glycoprotein and glycolipid have a number of similar properties. Further work will be required to resolve the detailed structures of the Thy-1 antigenic determinants of glycoproteins and glycolipids.

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