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THE ANTIGENIC AND FUNCTIONAL EXPRESSION OF GANGLIOSIDES IN THE PLASMA MEMBRANE OF HUMAN LYMPHOCYTES

The Ohio State University

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THE ANTIGENIC AND FUNCTIONAL EXPRESSION OF
GANGLIOSIDES IN THE PLASMA MEMBRANE OF
HUMAN LYMPHOCYTES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Charles Lawrence Hitchcock, B.S., M.A.

****
The Ohio State University
1981

Reading Committee:                      Approved By:
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Dr. Kenneth H. Jones
Dr. Thomas G. Hayes

Advisor
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Department of Anatomy
Gangliosides are a group of sialic acid containing glycolipid molecules located in the outer leaflet of a cell's plasma membrane. The experiments conducted for this study were designed to determine if these molecules are antigens on the surface of human lymphocytes and, if so, to determine their biochemical properties and role in binding interferon.

The experimental probe used for this study was rabbit antisera raised against the ganglioside GM1 extracted from normal human cerebral cortex. These antibodies demonstrated that populations of both T and B lymphocytes, obtained from adult human venous blood, express a GM1-like antigenic determinant on their surface. The percent of lymphocytes expressing this antigen on their surface decreases from approximately
40% in fetal cord blood to approximately 11% in adult peripheral blood. Thus indicating this antigenic determinant may also be a differentiation antigen for human lymphocyte subpopulations.

The G\(_{M1}\)-like antigenic determinant exhibits the biochemical properties and membrane behavior ascribed to gangliosides. The determinant is resistant to proteolytic digestion; whereas its expression is increased by treatment of cells with neuraminidase. Immunoprecipitation of radiolabeled membrane lysates indicates that the molecule(s) carrying the G\(_{M1}\)-like determinant is not a glycoprotein, but that it corresponds to \(^{3}H\)-G\(_{M1}\) incorporated into cell membranes and immunoprecipitated in an identical manner. The antigenic determinant, as well as exogenous G\(_{M1}\) incorporated into the plasma membrane, can undergo rapid cap formation. Anti-G\(_{M1}\) induced cap formation is both temperature and energy dependent, in addition to being dependent on an intact system of microfilaments. Cocapping studies indicate that this antigenic determinant is not associated with B\(_{2}\)-microglobulin, but that it is associated with both surface immunoglobulin and an Ia-like antigen containing molecule.

The ability of human fibroblast interferon to augment human natural killer (NK) cell activity was modulated by monovalent, Fab, antibodies to G\(_{M1}\). This modulation is both negative and positive, and varies with respect to the concentration of cells, Fab and interferon as well as demonstrating individual differences. These results, relative to NK cell activity, suggest that the G\(_{M1}\)-like antigenic determinant participates in the membrane receptor complex for fibroblast interferon.
ACKNOWLEDGEMENTS

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PART 1

INTRODUCTION

An essential property of cell membranes is their "sidedness". That is to say, cell membranes exhibit both structural and functional differences between inner and outer membrane surfaces. The expression of these differences is seen in unidirectional ion pumps, receptors for biologically active molecules, and in antigens which identify one cell from another. Sidedness arises from the asymmetric arrangement of membrane protein, lipids and associated carbohydrates (152). The fluid mosaic model of Singer and Nicolson (177) demonstrates this asymmetry by depicting the cell membrane as a sea of lipid with proteins of varying length floating in it. Another important feature of this model is that all plasma membrane associated carbohydrates are found on the external surface in the form of glycoproteins and glycolipids. As a group, these glycoconjugates form the glycocalyx and are responsible for many of the functions associated with cell membranes, such as cell-to-cell interaction and communication and specific binding of extracellular agents. These functions are altered or lost with the modification or loss of the saccharide portions of the glycoconjugate molecules.
A possible model for the various functions of membrane glycoconjugates is seen in a group of acidic glycolipids known as gangliosides. Gangliosides are a complex group of sialic acid (neuraminic acid) containing glycosphingolipids, primarily located in the eukaryotic cell's plasma membrane. Interest in the gangliosides stems from their proposed functions in: providing conformation for membrane glycoproteins (118,206), binding of various biologically active substances (23,52), immunoregulation (28,206), and in cell-to-cell contact (52,79). In addition, transformed cells (52,68) as well as subpopulations of hemopoietic (1,2) and lymphocytic cells (7,165,184) demonstrate characteristic patterns of gangliosides within their plasma membrane.

Gangliosides are amphipathic lipid molecules. They consist of both a hydrophobic ceramide portion and a hydrophilic, polar head group consisting of different saccharide residues (Figure 1 and Table 1). The ceramide portion consists of a sphingosine base joined by an amide bond to a fatty acid (23). The polar head group consists of several different carbohydrates including: glucose, galactose, N-acetyl-galactosamine, N-acetyl glucosamine and N-acyl-neuraminic acid (sialic acid). Whereas both neutral glycosphingolipids and gangliosides contain ceramide and carbohydrate containing polar head group portions, only gangliosides contain one or more sialic acid units in their carbohydrate chain (96). In man the only ganglioside-bound sialic acid is N-acetyl neuraminic acid, while N-glycolyneuraminic acid is found in other mammalian and lower vertebrate species (23).
New and novel gangliosides are being described at an ever increasing rate in the literature. The more "simple" gangliosides (Figure 1, Table 1), described in the recent past (52,204), consist of a variable number of sialic acid residues attached to either, or both, the terminal or internal galactose residues of the carbohydrate backbone of:

\[ \text{Gal(B1-4)GalNAc(B1-3)Gal(B1-4)Glc(B1-1)Ceramide} \]

or to each other in sialic acid-sialic acid units (96). Complex gangliosides include: gangliosides containing fucose in addition to the hexose and hexosamine units (96), macrogangliosides containing up to 19 carbohydrates (72), and branching gangliosides (202), in addition to simpler gangliosides, have recently been described in noneuronal cells.

Gangliosides are asymmetrically distributed in the plasma membrane where they are only located in the outer leaflet of the membrane. These molecules do not move perpendicular to the plane of the membrane, that is, the hydrophilic portion does not flip over into the hydrophobic region (172). However, ganglioside molecules are mobile within the plane of the membrane. Evidence which shows that gangliosides move laterally in artificial membranes (172), is seen in capping studies using cholera toxin (31,146,147) and antisera to gangliosides (35,169). Gangliosides added exogenously to cells and subsequently incorporated into the membrane have been shown to move laterally in the plane of the membrane (147,168). The ability of gangliosides to move laterally may
very well facilitate the molecular interactions required for the transduction of a signal from the external to the internal environment.

The carbohydrate chain does not remain free on the cell's surface, but tends to interact with carbohydrate chains from other glycolipids and glycoproteins (124,172,175). Intermolecular associations with other membrane glycoconjugates result in a localized clustering of gangliosides at the membrane surface. These intermolecular associations can arise from both hydrogen bonding and the formation of salt bridges in which a crosslinkage of sialic acid with other carbohydrates are formed by Ca\(^{+2}\) and/or Mg\(^{+2}\) ions (172,175). Such associations may occur at the surface of the glycocalyx or deep within this layer (118,206), and may result in gangliosides being "cryptic molecules" whose exposure varies with the cell cycle (1,64).

Cell surface carbohydrates are the primary site of a cell's interaction with its environment and the initial site of interaction with an external stimulus. The unique and diversified molecular configurations of the carbohydrate chains of glycosphingolipids, in general, allow these sugar components to have an important role in the recognition function of cells (192,193). Such structural diversity is exemplified by the observation that only one molecular configuration results when two amino acids combine, whereas two sugar molecules can join to form any one of sixteen possible molecular configurations (45). In the case of gangliosides, functional diversity parallels structural diversity.
Gangliosides are reported to function as receptors, alone or in conjunction with glycoproteins, for a variety of biologically active substances: These include a variety of biologically active substances: cholera toxin (34,185); tetanus toxin (185,196); C. botulinum neurotoxin (89); Grp. A Strep. exotoxin (158); lectins (104,179); serotonin (187,205); Sendai virus (71); thyrotropin (TSH) (115); luteinizing hormone (LH) (25); and interferons in both man and mice (6,13,14,198,199). Important to the role of gangliosides as receptor molecules is their association with membrane glycoproteins. Grollman et al. (66) concluded that the receptor for interferon and TSH consists of both a ganglioside and a glycoprotein component. The ability of gangliosides to move laterally in the plane of the membrane under physiological conditions is thought to lead to association of the ligand receptor complex with a specific membrane protein. For example, such an association with adenyl cyclase would result in signal transduction across the membrane (12). The ultimate result would be changes in cytoplasmic cAMP levels, and/or as changes in ionic flux across the membrane (66).

Several investigators have observed from their in vitro studies that gangliosides have a function in modulating the immune response. Miller and Esselman (49,110), using a murine system, have reported that ganglioside-containing liposomes or membrane derived vesicles can suppress B cell differentiation into plaque forming cells. Ryan and Shinitzky (153) found that gangliosides also can be mitogenic for murine
B cells. Yates et al. (207) studied a similar phenomenon using human peripheral blood mononuclear cells, and reported that a ganglioside mixture, as well as certain purified gangliosides, can inhibit, in a dose dependent manner, the cell response to nonspecific mitogens and allogeneic stimuli. Recent studies (Whisler-unreported results) showed that gangliosides also have the ability to modulate the response of natural killer cells to the effects of interferon. That this phenomenon may have physiological significance is derived from studies of the shedding of membrane vesicles as a random process for membrane turnover (45). Esselman and coworkers (28,49,55) have reported that antigen stimulated murine B cells release immunoregulatory substances into the culture media. These substances were identified as gangliosides by thin layer chromatography, and by the fact that anti-ganglioside serum abrogated their effect. Correa et al. (28) concluded that shed gangliosides modulate antigen competition by preventing antigen overload and directing B cell differentiation into plasma cells.

The amount of lipid associated sialic acid, and therefore by definition, the concentration of gangliosides, is highest in the brain (23). But gangliosides, as a group, are ubiquitous and have been found in the plasma membrane of all mammalian cells studied to date. The pattern of gangliosides in a given cell type will vary with the species, as will the pattern expressed on different cell types in the same species (23,117,118). Even though gangliosides are present they may not be exposed in such a way as to function as antigenic determinants on the
cell surface. Their antigenic expression may vary with cell cycle (64) and/or lie deep within the glycocalyx where their antigenic expression is sterically hindered by other carbohydrate residues. In support of this latter contention, studies using proteolytic enzymes (62,63,183), and neuraminidase (1,51,131,188) have reported an increase in both the functional and antigenic expression of plasma membrane gangliosides. There are also differences in both the qualitative and quantitative ganglioside patterns and antigenic expression associated with cell transformation (52,68,74,97,165) and the transforming agent (209). The site of change is at the level of specific glycosyltransferase activity. This can result in either an increase in the proportion of more simplified ganglioside, e.g. $G_m^2$, or the pattern may shift toward an increase in specific complex ganglioside, e.g. $G_{Tb}$. (68,209).

Investigators using both cholera toxin and antisera to gangliosides have concluded that gangliosides, as antigenic determinants, are differentially expressed on the surface of lymphocytes. In the mouse Stein-Douglas et al. (68,209) reported that $G_m^1$ was antigenically expressed on subpopulations of T and B cells as well as on thymocytes. Stein et al. (184) further concluded that "T and B cells differ in the accessibility of $G_m^1$ to antibody, and not necessarily in their content of $G_m^1". They also reported that affinity purified antibodies to asialo-$G_m^1$ (Figure 1) reacted only with mature T cells and a thymocyte subpopulation, but not with B cells. They concluded that the larger number and more diversified cell types which stained were the result of
crossreacting antibodies in their antibody preparation. Recently, asialo-G_{M1} has been described as an antigenic marker for murine pre-T cells (67), mature T cells (167), and natural killer (NK) cells (83,111,167,210). In addition, Arndt et al. (7) recently characterized their rat T-lymphocyte macrophage antigen (TLMA) as being asialo-G_{M1} and concluded that in the rat it serves as a differentiation marker for T cells. Macrogangliosides, expressing Ia-like antigenic determinates, have been reported as being differentially expressed on lymphocyte subpopulations in both mice and man (73,132). Ackerman et al. (1,2) were able to demonstrate that there are subpopulations of human lymphocytes and differentiating myeloid cells on the basis of cholera toxin binding per length of membrane surface. Schwarting and Marcus (165) reported that antiserum to G_{M1} or asialo-G_{M1} was capable of delineating subpopulations of human B and T cells, but were unable to ascribe a particular function or functions to them.

Interpretation of the results of studies on the antigenic expression of gangliosides are complicated by the multiple antigenic epitopes associated with the ganglioside's carbohydrate chain (55,110,201), as well as by the fact that these epitopes may be present on both glycoproteins and gangliosides (142). Tonegawa and Hakomori (190) showed that an antiserum specific for the ganglioside G_{M1} or asialo G_{M2} would precipitate up to five glycoproteins, in addition to the specific ganglioside from labeled 3T3 Balb/c and 3T3 KiMSV transformed cell lines. Recent studies by Esselman and associates
(55,110,201) have supported their earlier contention that a G\textsubscript{M\textsubscript{1}}-like ganglioside shares one or more epitopes with the Thy-1 glycoprotein molecule found on murine T cells. A similar finding has been made in humans where antisera to a carbohydrate-defined Ia antigen, (possibly a macroganglioside), and to the DRW glycoproteins reciprocally block the binding of the other, and cocap (132,155). The blood group I-i antigens are expressed on both macrogangliosides and secreted glycoproteins (51,202). I-i antigen activities are associated with the carbohydrate sequence of both molecules.

Cell-to-cell contact is a fundamental process for cellular recognition in embryogenesis and in the immune response. This cellular adhesion is probably mediated by components of the glycocalyx, and may include gangliosides (52,68). Huang (79) reported that nonadhesive chicken erythrocytes would adhere to HeLa cell monolayers when gangliosides are incorporated into their plasma membrane. Obata et al. (126) reported that G\textsubscript{M\textsubscript{1}}, when added to the culture medium, would promote neuromuscular junction formation between myotubes and spinal cord explants. In the case of fusing and nonfusing myoblast cell lines, G\textsubscript{D\textsubscript{1a}} was found in the plasma membrane of fusing cells but not in the nonfusing mutants (23). Hakomori (68) and Fishman and Brady (52) reviewed the fact that ganglioside patterns differ between transformed and nontransformed cells, and that upon temperature specific mutant reversion the original ganglioside pattern returns. One example is the synthesis of G\textsubscript{D\textsubscript{1a}} at very early stages of cell-to-cell contact in
nontransformed cells, which is not seen in transformed cells. Cell-to-cell contact may be mediated by an enzyme-substrate like interaction (68). Two cells would interact with one another when a glycosyl transferase, or a series of them, on cell A bound to a carbohydrate chain on the surface of cell B. The glycosyl transferase could use the sugar molecules of this chain as an acceptor molecule and extend its length by the sequential addition of monosaccharide units to its terminal end. It might also be possible for the carbohydrate chain on the surface of cell B to serve as a source of monosaccharides for subsequent carbohydrate chain elongation on cell A glycoconjugates. Such a mechanism has been proposed for the Ir gene restrictions associated with lymphocyte interactions during an immune response (16), and has recently been proposed by Parish and McKenzie (132) as the relationship between protein Ia antigens (enzyme) and the macroganglioside Ia antigens (substrate).

This dissertation has been designed to address the following questions:

1. Are gangliosides, or related glycoconjugates, present as antigenic determinants on the surface of human lymphocytes?

If the answer to question number one is yes, then:

2. Are these molecules differentiation antigens?

3. What are the properties of these molecules in the cell
membrane? For example: can they be capped alone or in conjunction with other known membrane components? Are they susceptible to enzyme modification? Can they be radiolabelled and immunoprecipitated?

4. Do gangliosides have a function in the plasma membrane of human lymphocytes?

Different probes have been used to study gangliosides in the plasma membrane of lymphocytes of various species including man. These probes include: antibodies specific for gangliosides (119,184), cholera toxin (1,2,146,147), labeled gangliosides taken up and incorporated into the plasma membrane (168,179), as well as biochemical analysis (103,167,182). In order to answer the above questions I chose to use a rabbit antiserum to the ganglioside GM1, purified by affinity chromatography, as the probe for this study. Antibody molecules were considered to be a physiological probe with possible clinical significance. This latter point is important in light of recent findings of antibodies to gangliosides present in certain autoimmune disease states (8,75,156). The bivalency of the molecule allowed me to study ganglioside capping in the plane of the membrane; whereas, a monovalent preparation facilitated the study of ganglioside receptor functions. The heterospecific nature of the serum allowed for maximum recognition of antigen epitopes associated with the ganglioside's carbohydrate chain.
**MATERIALS AND METHODS**

**Animal and Cell Sources**

Female, NZW rabbits weighing between 4 and 9 pounds were purchased through the Laboratory Animal Center (The Ohio State University). All animals were housed, fed, and given water ad libitum in the animal facility in Wiseman Hall.

Normal, healthy adult humans, with no recent history of medication, served as the source of peripheral blood, obtained by vena puncture. The Department of Obstetrics and Gynecology, The Ohio State University, provided the fetal cord blood obtained from postnatal umbilical cords. Thymus tissue was obtained from children, less than 3 years of age, undergoing open heart surgery. Spleen tissue was obtained by the Office of Tumor Procurement, The Ohio State University.

**Chemicals**

Normal human brain gangliosides were obtained from Dr. Allan Yates, Department of Pathology, The Ohio State University, and included the pure forms: $G_{M1}$, $G_{M2}$, $G_{M3}$, $G_{M4}$, $G_{T1}$, $G_{DA}$, and $G_{DB}$ (Table 1) as well as a mixture of pure gangliosides. The cholesterol and egg lecithin were from Supelco, Inc. (Bellefonte, PA). Bacto complete adjuvant H37Ra and incomplete Freund adjuvant were obtained from DIFCO Laboratories (Detroit, MI). The goat anti-rabbit whole serum, fluorescein (FITC)
labeled F(ab')$_2$ fragment of goat anti-rabbit IgG F(ab')$_2$ fragment, goat anti-rabbit IgM (heavy chain specific), goat anti-rabbit IgG (Fc fragment specific), F(ab')$_2$ fragment of goat anti-rabbit IgG (heavy and light chain specific), rabbit IgG (purified chromatographically) and rhodamine (TRITC) labeled goat anti-mouse IgG (heavy and light chain specific) were obtained from Cappel Laboratories, Inc. (Cochranville, PA). Mouse monoclonal IgG$_{2a}$ antibody (clone 26/114 HLK) to human B$_2$-microglobulin and guinea pig serum (Hemo—Lo) were from Accurate Chemical and Scientific Corp. (Hicksville, NY). The mouse monoclonal antibody to human Ia, Aquasol-2, tritiated sodium borohydride (25 mCi) and carrier free iodine-125 (100 mCi/ml) were purchased from New England Nuclear (Boston, MA). Rhodamine (TRITC) labeled goat anti-human total immunoglobulins were purchased from Kallestad (Chaska, MN). RPMI 1640 medium (with L-glutamine), fetal bovine serum (FCS), Seligmann's balanced salt solution (SBSS), and colchicine were from GIBCO (Grand Island, NY). The 2,4-dinitrophenol, E-aminon-caproic acid, sodium m-periodate, 2-deoxy-D-glucose, sodium fluoride, ethylenediamine tetracetic acid-tetrasodium salt (EDTA), phenylmethyl sulfonyl fluoride (PMSF), cycloheximide, dimethyl sulfoxide (DMSO), methylated bovine albumin (mBSA), papain, B-glucosidase, trypsin inhibitor (soybean), B-galactosidase (Grade VIII), galactose oxidase (Type IV), DNase I, pepsin, lactoperoxidase, protease (Type V), and trypsin (Type IX) were obtained from Sigma Chemical Co. (St. Louis, MO). The cytochalasin B, neuraminidase (Vibrio cholerae) 500 units per ml (VCN), bovine albumin (BSA) and Staphylococcus aureus (PANSORBIN) as a source of Staph protein A (SPA) were from Calbiochem-Behring Corp. (LaJolla, CA). The
Sepharose 4B Cyanogen bromide-activated and Sephacryl S200 were from Pharmacia Fine Chemicals (Piscatany, NJ). Normal human serum albumin, 25%, was from Travenol Laboratories, Inc. (Glendale, CA). Nonidet-40 (NP-40) was obtained from BDH Chemicals Ltd. (Poole, England). The 3-aminopropyltriethoxysilane (Lot 4-1336) was from Polysciences, Inc. (Warrington, PA). Sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylene-bis-acrylamide (BIS), N,N,N',N'-tetramethylenediamine (TEMED), Coomassie brilliant blue R-250 dye, dithiothreitol (DTT), ammonium persulfate, tris (hydroxymethyl) aminomethane (TRIS), high and low molecular weight standards for SDS-PAGE, DEAE Bio-Gel A (DEAE) and CM Bio-Gel A (CM) were obtained from Bio-Rad Laboratories (Richmond, CA). Ficoll-Hypaque (24:10) was provided by the laboratory. Phenol reagent and all organic solvents were obtained from The Ohio State University Laboratory Stores.

**Antisera Production**

Rabbit antiserum to gangliosides was produced according to the protocol of Ohsawa and Nagai (127) (Appendix A). Mixed lipid micelles consisting of a 1:4:10 (w/w/w) ratio of GM1, egg lecithin and cholesterol, for the initial injection, were prepared as follows: 6 mg of GM1 in chloroform, was added to 25 mg of egg lecithin and 60 mg of cholesterol and after the addition of 2.0 ml of redistilled chloroform, the lipids were dried down at 40°C under a steady stream of N2 (N-EVAP, Organomation Associates, Inc., Northborough, MA). The lipids were then dissolved in 1.0 ml of tetrahydrofuran (THF) and added to 19 ml of saline, mixed on a vortex, and the resulting micelles were obtained by
The micelles were resuspended in 3.0 ml of a 1.0% on BSA in PBS solution and allowed to cure for up to 4 days at 4°C. An injection emulsion was prepared by rapidly injecting the micelle preparation into another syringe containing 3.0 ml of complete adjuvant H37Ra. Each rabbit was injected with 2.0 ml of this emulsion, containing 2.0 mg of the ganglioside, by injection into the four foot pads and subcutaneously. Booster injections were given subcutaneously and contained 1.0mg GM1 in a similar emulsion. Boosters injections were given 2 and 4 weeks after the initial injection and at variable times thereafter in an attempt to increase the antiserum titer.

Rabbits were bled on a periodic basis from the central ear vein or exsanguinated by cardiac puncture. Blood was collected in a 50 cc centrifuge tube, heated for 1 hour at 37°C and allowed to clot overnight at 4°C. The resulting serum was separated from the clot by centrifugation and heated at 56°C for 30 minutes in order to inactivate the serum's components of complement. This final heat inactivated serum was then stored at -20°C.
**Antiserum Fractionation**

The gammaglobulin fraction (Ig) was prepared by adding an equal volume of a saturated ammonium sulfate (NH$_4$SO$_4$) solution to a serum sample with constant vortexing. The resulting precipitate was washed twice with 50% NH$_4$SO$_4$ and redissolved in a volume of H$_2$O equal to one half of the original serum volume. The final Ig fraction was desalted by dialysis; 48 hours with 3 changes at a 1:500 ratio of sample to saline or 0.01M phosphate buffer (pH 8.0).

The IgG components of the gammaglobulin fraction were obtained by DEAE ion exchange chromatography (180). To a DEAE-Bio-Gel A column (2.5 cm x 25 cm), equilibrated with 0.01 M phosphate buffer pH 8.0, was added 15 ml of an Ig sample previously dialyzed against the column buffer and clarified by centrifugation at 10,000 x g for 20 minutes. The IgG peak, the first peak eluted, was back concentrated, dialyzed against SBSS or saline and stored at -20°C. The serum components binding to the column were eluted with 0.01 M phosphate - 0.3 M sodium chloride buffer (pH 8.0), back concentrated and dialyzed overnight against a buffer containing 0.01 M phosphate - 0.3 M sodium chloride (pH 7.2). The column was re-equilibrated with the starting buffer as determined by eluate conductivity. The IgM antibodies were obtained by gel chromatography on a 2.5 x 128 cm column of Sepharcyl S200 equilibrated with a pH7.2 0.01 M phosphate - 0.3 M sodium chloride buffer. A total of 6.0 ml of the sample was applied to the column and 4.0 ml fractions were collected. The IgM containing peak was back concentrated and dialyzed against saline and stored at -20°C.
Antiserum Affinity Purification

Antibodies directed against BSA were removed from serum samples by affinity chromatography on a BSA-Sepharose 4B column. The column gel was prepared according to the instruction insert accompanying the CNBr-activated Sepharose 4B. To the PBS equilibrated affinity column (24 x 0.9 cm) was added a 12 ml serum sample. The sample flowed through the column at 1 x g and was washed with PBS until the % absorbance was less than 2% (UVICORD 5 LKB Instruments, Stockholm, Sweden). The anti-BSA bound to the gel was eluted with a 0.1 M glycine-HCl buffer (pH 2.5), and the column re-equilibrated with PBS as determined by the conductivity of the eluate.

Purification of antibodies to G\textsubscript{M1} by affinity chromatography, (Appendix B), was accomplished by the addition of a serum sample on to a G\textsubscript{M1}-aminopropyl silica gel column (6 x .9 cm) (92,93). A 30 - 50 ml, clarified, sample of serum was diluted 1:1 with the initial column buffer (containing .1 M Tris, .1 M sodium chloride and 0.01 M EDTA, pH 7.3). The sample was applied to the column by a Holter pump (Extracorporeal Medical Specialities, Inc., King of Russia, PA) at a flow rate of 0.5 to 0.7 ml per minute. The column was flushed with the starting buffer until the absorbance at 280 nm was less than 0.02. The column bound anti-G\textsubscript{M1} antibodies were then eluted with 50 ml of 2 M potassium thiocyanate in PBS (pH 7.3). The eluate was back concentrated by ultratfiltration and dialyzed against 2 changes of PBS. The desalted affinity purified anti-G\textsubscript{M1} antibodies were clarified by ultracentrifugation at 100,000 x g for 60 min. and stored at -80 °C.
reactivity studies, 0.146 nM of ganglioside per ml of VBSG was used, and the liposomes were prepared in an identical manner. The gangliosides used were $G_m^2$, $G_m^3$, $G_m^4$, $G_{D1a}$, $G_{D1b}$ and $G_T1$ (Table 1) in addition to galactocerebroside (P-L Biochemicals, Milwaukee, WI).

Identification of the constituents in a given antiserum sample was accomplished by immunoelectrophoresis (IEP) as described by Hudson and Hay (80). Microscope slides were coated with an agarose-barbitol solution containing 1% agarose in barbitol buffer (each liter containing 0.92 gm diethyl barbituric acid and 5.15 gm sodium diethyl barbiturate) and the IEP patterns cut. Approximately 10 ul of sample in the upper well and a control normal rabbit serum containing bromophenol blue as a tracking dye in the lower well were electrophoresed until the dye front was within 1 cm of the slide's end. The appropriate goat antiserum, to whole rabbit serum or to purified immunoglobulin fractions, was placed in the trough and immunodiffusion was allowed to progress overnight.

The Lowry method (102) was used for the determination of sample protein concentration. A 1.0 mg per ml solution of ovalbumin was used as the reference protein. The standard curve was generated using 10 ul, 20 ul, 40 ul, 80 ul and 100 ul of the standard protein. Either 10 ul of undiluted or of a 1:10 dilution of the sample was used for each test. All standards and tests were performed in duplicate. The volume of each tube was brought to 200 ul with distilled water and 1.0 ml of solution A (1.0 ml 1% sodium tartrate, 1.0 ml 1% cupric sulfate mixed together in 50 ul of 2% sodium carbonate in 0.1 N sodium hydroxide). After vortexing, followed by 10 minutes of incubation, 100 ul of a 1:1 Folin
The affinity column washed with 4 M guanidine hydrochloride and re-equilibrated with the starting buffer.

**Antisera Characterization**

The microcomplement fixation assay described by Milewicz, et al. (109) was used to determine anti-\(G_{M1}\) titer and serum reactivity with gangliosides other than \(G_{M1}\) (Appendix C). A veronal buffer (VBSG), containing 0.15 mM CaCl\(_2\), 0.5 mM MgSO\(_4\) and 1.0% gelatin (pH 7.4) was used for all dilutions. Each test consisted of 200 ul antiserum, 200 ul antigen, 200 ul guinea pig serum (diluted to a concentration resulting in 80% hemolysis of sensitized sheep red cells), and 600 ul VBSG. Following an 18- to 24-hour incubation at 4°C, 200 ul of a 50 x 10\(^6\) per ml solution of sensitized sheep red cells were added and further incubated for 60 minutes at 37°C. The hemolysis was stopped by centrifugation at 1500 rpm for 10 minutes at 4°C, and the degree of complement mediated hemolysis was measured by determining the amount of hemoglobin released by the absorbance of the supernate at 413 nm (Beckman DU8 spectrophotometer). The antigen solution of ganglioside-liposomes was prepared as described by Naiki, et al. (119), and consisted of a 1:2:10 ratio (w/w/w) of ganglioside, egg lecithin and cholesterol. The liposomes were made by combining and then drying down 20 ug \(G_{M1}\), 40 ug egg lecithin and 200 ug cholesterol and resuspending them in 10 ml of absolute ethanol followed by vortexing and sonication for 10 minutes. For the assay this solution was diluted 1:10 with VBSG and sonicated, which resulted in 40 ng of G\(_{M1}\) per test. For the cross
reagent (OSU Lab. Stores) was added to each tube, vortexed and incubated for 30 minutes. The absorbance at 700 nm of each tube was read, standard curve constructed and the sample protein concentration calculated with the aid of a Texas Instruments TI 55 calculator preprogrammed for linear regression analysis.

**Cell Handling**

Mononuclear cell preparations from peripheral blood (PBMC), fetal cord blood (FBMC), thymus and spleen were obtained by a 25-minute centrifugation at 1650 rpm over a Ficoll-Hypaque gradient. The resulting cells taken from the interface had greater than 90% viability as measured by trypan blue exclusion. Cells were further separated by passing the cells through nylon wool columns, culturing on plastic petri dishes or by formation of rosettes with neuraminidase pretreated sheep erythrocytes.

Up to $1 \times 10^8$ mononuclear cells in 5 ml of RPMI 1640-10% human serum (RPMI-HS) medium were added to a nylon wool column (10 cc plastic syringe firmly packed with washed nylon wool), pre-equilibrated with SBSS-0.5% human serum albumin (SBSS-HSA). After a 30 minute incubation at 37°C and in 5% CO$_2$, the nonadherent cells, enriched for T cells and null cells, were removed by flushing the column with 100 ml of SBSS-HSA. The adherent cells, mainly B cells and monocytes, were removed by alternating flushing with 42°C preheated RPMI - 0.5% human serum albumin and plungering. The cell viability was greater than 80% for each fraction.
Monocytes were removed by culturing mononuclear cells on petri dishes for 2 hours at 37°C and in 5% CO₂. Nonadherent cells were recovered by vigorously washing the plates with SBSS - 5% human serum, preheated to 37°C. Nonadherent cell viability was greater than 90%.

Mononuclear cell preparations were enriched for T cells by rosette formation (E rosettes) with neuraminidase pretreated sheep red blood cells (SRBCs). To 500 ul of PBMCs, 15 - 20 x 10⁶ per ml in RPMI, was added 500 ul of SRBC, previously pretreated with neuraminidase at 5 units per 1 x 10⁶ cells and used at a concentration of 4.5 - 5 x 10⁸ per ml in RPMI, and 2 drops of human serum absorbed against SRBCs. Following a 15 minute incubation at 37°C, the cells were spun at 500 rpm for 5 minutes and cooled at 4°C for 2 to 4 hours. The cells were gently resuspended, pipetted over 3 ml of Ficoll-Hypaque and centrifuged for 30 minutes at 1000 rpm. The E rosette forming T cells were found in the pellet and the B cells at the interface. The SRBCs were lysed by osmotic shock and the T cells washed with SBSS. Cell viability was greater than 85%.

**Indirect Immunofluorescent Staining**

The antigenic expression of GM₁ or GM₁-like molecules on the surface of human mononuclear cells was demonstrated by an indirect immunofluorescent technique. All antisera were periodically clarified by ultracentrifugation at 100,000 x g for 60 minutes. Cells were washed 2 times in SBSS, counted, and 1 - 2 x 10⁶ cells pipetted into Fisher Tubes (404-978-145 Fisher Scientific). The cells were washed twice with
SBSS, precooled to 4°C, and the pellet gently resuspended in the first antiserum. The amount of antiserum added varied with protein concentration and not dilution. Initial antiserum binding was allowed to proceed for 60 minutes at 0°-4°C and the cells subsequently washed 3 times in 4°C SBSS. Antibodies to G_{M_1} were demonstrated by staining the cells with 100 ul of a 1:30 dilution of FITC labeled-F(ab')_2 fragment of goat anti-rabbit IgG F(ab')_2 fragment for 60 minutes at 0°-4°C. Excess second antiserum was removed by washing the cells 3 times with 0°-4°C SBSS. The cells were fixed for 5 minutes with 4% formaldehyde in SBSS and washed twice with SBSS. The cells were stored at 4°C for up to 48 hours prior to examination. Between 200 and 600 cells per test were counted.

The cells were examined with a Zeiss Epi-Fluorescence microscope equipped with phase contrast and the ability to examine both rhodamine and fluorescein labeled cells. All cells were examined under oil with a Plan 100 x phase objective lens. All photomicrographs were made on TRIX-Pan 35 mm film using a Zeiss MC 35 camera and an MC 63 automatic exposure control.

Enzyme Pretreatment of Cells

Mononuclear cell suspensions were obtained from adult peripheral blood, fetal cord blood, adult spleen and infant thymus as described earlier. Prior to enzymatic pretreatment, cells were washed three times in SBSS and adjusted to the appropriate cell concentration for each experiment.
Cells in 1.0 ml of SBSS, were incubated with Vibrio cholerae neuraminidase (VCN) at a concentration of 10 units per 5 x 10⁶ cells, for 30 minutes at room temperature or 37°C. (There is no apparent difference in enzyme activity when incubations are performed at either temperature). In order to prevent cell clumping, the VCN pretreated cell were washed once in RPMI 1640 medium containing 10% FCS, and subsequently washed three times in SBSS precooled to 4°C.

Proteolytic enzyme pretreatment of cells was performed by the addition of 100 or 200 ul of an appropriate enzyme solution to 10 x 10⁶ cells in 500 ul of SBSS. Enzyme solutions were prepared by adding either trypsin, at a concentration of 1.0 mg/ml, or protease, at a concentration of 1.25 mg/ml, to SBSS and adjusting the pH to 7.2. In order to reduce cell clumping, DNAase was added to each enzyme solution, to yield a final concentration of 1.0 mg/ml. In experiments where protease was used, this solution was preheated to 37°C for 90 minutes prior to its addition to the cells. Following an incubation at 37°C for 30 minutes, the reaction was stopped by the addition of RPMI 1640 - 10% FCS medium. After a 10 minute incubation, the cells were centrifuged and washed three times with 4°C SBSS.

Modification of cell surface carbohydrate residues was accomplished by resuspending 5 x 10⁶ cells in 1.0 ml of SBSS containing 1.0 mg of B-galactosidase, 1.0 mg of B-glucosidase or 1.2 mM NaIO₄. Enzymatic cleavage of carbohydrate residues was performed at 37°C for 30 minutes, whereas sialic acid oxidation occurred at 0°-4°C for 15 minutes in the NaIO₄ solution. Following pretreatment, the cells were washed three
times in SBSS at 4°C.

**Ganglioside Pretreatment of Cells**

The gangliosides, G_{M1} or G_{T1b}, at a concentration of 10 or 20 nM per 1 x 10^6 cells, were dried down under N_2, resuspended in 500 ul of SBSS, vortexed, and sonicated for 10 minutes. The resulting micellar solution of gangliosides was added to a 500 ul suspension of cells in SBSS. The mixture was incubated for 60 minutes at 37°C, after which the cells were washed three times with SBSS precooled to 4°C.

**Capping Inhibition**

PBMCs, pretreated with SBSS, VCN or G_{M1} were washed 3 times with SBSS prior to washing with a pharmacologic reagent. Cytochalasin B (Cyto. B-10ug/ml) was dissolved in DMSO, to a concentration of 500 ug/ml, and diluted to 10 ug/ml with SBSS just prior to use. Sodium azide (NaN_3 - 0.01M), sodium fluoride (NaF - 0.1M), 2,4 dinitrophenol (DNP - 5x10^{-4}M), colchicine (Col - 10^{-5}M), lidocaine 2-deoxy-D-glucose (0.05M), and cycloheximide (10ug/ml) were prepared with SBSS, adjusted to pH 7.2 and stored at 4°C. Washed cells, 1 - 2 x 10^6, were incubated first for 30 minutes at 37°C and then at 4°C for an additional 30 minutes in 200 ul of the respective media. The cells were spun and resuspended in 100 ul of antiserum and 200 ul of the appropriate inhibitor and incubated for 60 minutes on ice. The cells were washed 3 times and incubated with the FITC-labeled second antibody for 60 minutes on ice. After washing, the cells were resuspended in 200 ul of the
respective agent and incubated for 10 to 15 minutes at 37°C. Capping was stopped by the addition of 200 µl of 4% formaldehyde in SBSS. After 5 minutes of fixation, the cells were washed and examined under oil. Cells considered as capping had staining at one pole and occupied no more than 1/3 of the cell surface. Cytoplasmic staining which was localized at one pole was also considered as being capped.

Cocapping

In addition to determining if Gm1 cocaps with a known membrane protein or glycoprotein, experiments were designed to look at possible steric hinderance of second test antiserum binding by the first antiserum already bound to the cell surface. Therefore experimental protocols varied with the antisera used and the questions being asked. PBMCs, unfractionated or the nylon wool adherent fraction, were pretreated with SBSS or VCN, washed and dispensed at 1 - 2 x 10^6 cells per tube. Mouse monoclonal antisera to human B2-microglobulin or Ia antigen were used at a 1:20 or 1:100 dilution respectively. Rhodamine labeled F(ab')2-goat anti-human total immunoglobulins (TRITC anti-mIg) was used at a 1:10 dilution, while 50 or 100 µg of affinity purified anti-Gm1 serum (16P4), was used in each experiment.

Capping of membrane immunoglobulins (mIg) was accomplished by first reacting the cells for 30 minutes at 4°C with the TRITC anti-mIg and then for 15 to 30 minutes at 37°C. Capping was then stopped by fixation, the cells washed and stained for Gm1 (see above). In a second series of experiments cells were first reacted with 16P4, washed and
stained as previously noted and then treated with TRITC anti-mIg for 60 minutes. The entire procedure was done on ice and the cells were allowed to cap while being studied under the microscope.

Capping of B₂-microglobulin and Ia antigen was induced on cells, previously treated for 30 to 60 minutes at 4°C, with the appropriate antiserum, and a 1:10 dilution of rhodamine labeled goat anti-mouse IgG antiserum. The labeled antiserum was allowed to bind for 30 minutes at 4°C before inducing capping by incubating the cells for 15 to 30 minutes at 37°C. Capping was stopped by fixation. Capping of GmI was accomplished in a similar manner. The second test antiserum was reacted with the cells for 30 or 60 minutes at 4°C and after washing, stained with the appropriate labeled antiserum for 30 or 60 minutes.

Cell Surface Labeling

**Glycoconjugate Tritiation**

Cell surface glycoproteins and glycolipids were labeled with tritium, according to the methods of Gahmberg and co-workers (62,63,64). Exposed glycoconjugates were oxidized by pretreatment of 20 - 30 x 10⁶ PBMCs in 1.0 ml PBS, by one of three methods, followed by reduction with tritiated sodium borohydride (NaB³H₄). Sialic acid oxidation was accomplished by adding 1.0 ml of iced 4mM NaIO₄ in PBS to the cell suspension. After a 10 minute incubation on ice, while in the dark, the reaction was stopped by adding of 100 ul of PBS containing 0.1 M glycerol. Galactose and galactosamine residues were oxidized with galactose oxidase, alone or in combination with VCN. To the cell
suspension, was added 5 units of galactose oxidase alone or in combination with 12.5 units of VCN. The resulting mixture was then incubated for 60 minutes at 37°C. After washing three times with PBS, the pretreated cells were resuspended in 4.0 ml PBS and 0.5 mCi NaB\(^{3}\)H\(_{4}\) added. After a 30 minute incubation at room temperature, the cells were washed four times in PBS and resuspended in 200 ul of PBS and stored on ice until lysed.

**Cell Uptake of Tritiated G\(_{50}I\)**

A total of 300 nm of tritiated G\(_{50}I\) (\(^{3}\)H-G\(_{50}I\)), obtained from Dr. Allan Yates, was dried down under N\(_{2}\), resuspended in 2.0 ml of SBSS and sonicated for 10 minutes. The resulting \(^{3}\)H-G\(_{50}I\) micelles were added to 120 x 10\(^{5}\) PBMCs in 2.0 ml SBSS. The cell suspension was incubated for 3 hours at 37°C and under 5.0% CO\(_{2}\)-Air. The unincorporated \(^{3}\)H-G\(_{50}I\) was removed by washing the cells four times with SBSS. The washed \(^{3}\)H-G\(_{50}I\) labeled PBMCs were resuspended in 1.0 ml SBSS and kept on ice until lysis.

**Cell Membrane Radiiodination**

Radiiodination of cell membrane components was accomplished with the use of lactoperoxidase and H\(_{2}\)O\(_{2}\) as catalysts. To 20 x 10\(^{6}\) PBMCs, suspended in 200 ul of PBS was added, in order, 10 ul of \(^{125}\)I and 20 ul of PBS containing 0.03% H\(_{2}\)O\(_{2}\). The cell suspension was mixed and allowed to stand for 5 minutes. After this time an additional 75 ul of lactoperoxidase, and 20 ul of PBS-H\(_{2}\)O\(_{2}\) was added. Following a 5 minute
incubation, an additional 20 ul of the 0.03% H₂O₂ was added and the resulting mixture further incubated for 15 minutes. All incubations were done in a fume hood at room temperature. The excess reactants were removed by washing the cells four times with iced PBS, and finally resuspending the cells in 100 ul of PBS and stored on ice until lysis.

**Cell Lysis**

Labeled cells were lysed by the addition of an equal volume of a solubilizing solution [1.0% NP 40 (V/V), 1% (W/V) soy bean trypsin inhibitor, 2.0% (W/V) E-amino caproic acid, and 2mm phenylmethyl sulfonylfluoride], mixed, and incubated on ice for 15 minutes. Cell debris and nuclei were removed by centrifugation at 1000 x g for 20 minutes at 4°C. Unless immediately used, the supernatant was carefully removed and stored at -20°C.

**Immunoprecipitation**

A modification of the procedure of Tonegawa and Hakomori (190) was used for the immunoprecipitation of the radiolabelled cell lysates. To a lysate sample containing 1-1.5 x 10⁶ cpm was added 1.0 mg of an antiserum; 16P₄IgG or NRS IgG, or 25 ul of PBS. This mixture was incubated overnight at 4°C. To each sample was added 100 ul of a 10% suspension of protein A containing Staph aureus (SPA-'PANSODREBIN'-Calbiochem) in PBS and incubated overnight at 4°C. The lysate-SPA mixture was spun at 10,000 x g for 10 minutes. The resulting pellet was washed two times with PBS and resuspended in
50-100 ul of SDS-electrophoresis buffer containing 6M urea and 0.02M dithiollthreitol (DTT). The pellet suspension was heated for 5 minutes at 100°C followed by a 10,000 x g centrifugation for 10 minutes. High and low molecular weight standards were also reduced with the same solution and heated at 100°C for 5 minutes. The resulting supernatants were then ready for SDS-polyacrylamide gel electrophoresis.

**SDS-Polyacrylamide Electrophoresis**

Disc gel electrophoresis was performed in much the same manner as described by Fairbanks et al. (50), and with the aid of a Bio-Rad disc gel electrophoresis unit. Cylindrical gels, 5.5 mm x 125mm, of 7.5% acrylamide were used for these studies. In order to form a sharp interface at the gel surface, degassed, distilled water was carefully pipetted onto the surface of the unpolymerized gel. The gels were allowed to polymerize, and were stored overnight in SDS-electrophoresis buffer prior to use.

To 60 ul of each sample and molecular weight standards was added 20 ul of a 40% sucrose-SDS electrophoresis buffer solution containing 1 mg/ml of bromophenol blue dye. The samples were stacked onto the gel surface by an initial electrophoresis at 1 amp per tube for 30 minutes. After this time the sample has penetrated the gel and the amperage was increased to 8 amps per tube. A LKB 2130 constant power supply was used for all electrophoresis runs. Electrophoresis was continued until the dye front was within 1.0 cm of the bottom of the gel.
Sample gels were removed, the dye front marked with india ink, and quickly frozen in a dry ice-isopropanol bath. A 10% acetic acid solution was used to thaw each gel to the point where it could be sliced with a Bio Rad 190 gel slicer. Gels were cut into approximately 1mm thick disc. Each disc from $^{125}$I labelled samples were placed into 12cm x 75mm glass tubes, stoppered and the gamma emissions were counted on a Beckman 4000 Gamma Counter. Gel discs of tritiated samples were placed into minivials containing 4-5ml of Aquasol-2 scintillation cocktail and counted on a Beckman LS 7000 Scintillation Counter. The counting efficiency was approximately 50%. Data is expressed as the percentage of total counts added to the gel that is found in each disc, as compared to its relative mobility (Rf) in the gel. Gels of molecular weight standards were stained in Fairbanks solution (10% acetic acid, 20% isopropanol and 0.005% Commassie blue dye) overnight, and destained in 10% acetic acid. The position of each band in the gel as it compared to the position of the original front, was determined and Rf value for each molecular weight standard calculated.
Antibody Specificity

Antibodies to G\textsubscript{M1} appeared within 4 to 6 weeks after the initial antigenic challenge and serum titers were maintained for several months. In an attempt to affinity purify the antibodies to G\textsubscript{M1}, serum was passed through a G\textsubscript{M1}-immunoabsorbent column and eluted with 2M KSCN in PBS as the chaotroic agent (93). Specificity of the antisera was determined by complement fixation using mixed micelles of ganglioside, egg lecithin and cholesterol, in a 1:2:10 w/w/w ratio (109). The titer of each antiserum was calculated as the highest dilution of serum that fixed 50% of the complement present. In order to equate the various antisera with one another, the antiserum activity is expressed as ug protein resulting in 50% lysis (Table 2).

The ability of affinity chromatography to concentrate specific antibodies is also expressed in Table 2. Affinity purified antisera has an increase in antibody activity, as compared to preadsorbed and unadsorbed serum, ranging from 10 to 100 fold. Immunoelectrophoresis of the column bound and eluted material (Plate I) shows that the major antibody bound is an IgG molecule which exhibits a retarded electrophoretic mobility relative to unbound IgG. Very little IgM was eluted from the column. Nonimmunoglobulin serum components were also
seen to bind to the immobilized G\textsubscript{M1} and eluted along with the antibodies. This is in contrast to the findings of Kundu and Roy (93), but in accordance with Chambers et al. (submitted for publication) who found that serum albumin has binding sites for G\textsubscript{M1}.

The antibody activity of affinity purified sera to gangliosides other than G\textsubscript{M1} and G\textsubscript{D1b} is seen to vary with each serum examined. As with antibody to G\textsubscript{M1}, the presence of crossreacting antibodies is considered to be a function of individual differences among the animals as well as a property of a given antiserum pool. The presence of crossreacting IgM antibodies has been associated with antisera to G\textsubscript{M1} (107,119), and would explain slight to high levels of crossreactivity to G\textsubscript{M2} and G\textsubscript{D1a} seen with the affinity purified antisera. The relative lack of crossreactivity in IgG fractions of this sera is again in accord with other observations (107). The high antibody activity to G\textsubscript{D1b} (Table 2) and to asialo-G\textsubscript{M1} (Table 1), not used in this study, have been noted as a common feature of both IgG and IgM fractions of anti-G\textsubscript{M1} sera (93,119). There is as yet no known explanation beyond the fact that these heterogenous antibodies recognize more than one antigenic epitope and that G\textsubscript{M1} shares antigenic epitopes with both G\textsubscript{D1b} and asialo G\textsubscript{M1} to a larger degree than with G\textsubscript{M2} or G\textsubscript{D1a}.

Tables 3 and 4 represent the results of a second approach in determining antiserum specificity. In these sets of experiments, an antiserum dilution was chosen which was approximately one-half the antiserum’s titer. The antiserum dilution was held constant and the amount of antigen per tube was varied. This is the opposite of the test conditions used for Table 2. IgG antibodies exhibit less
crossreactivity as compared to whole serum. Even at the highest antigen concentration the degree of crossreactivity, as expressed by percent complement fixation, is less in the IgG fraction than in the whole serum. Serum antibodies react equally well with GM1 (Table 3). The differences in crossreactivity between whole serum and IgG is again attributable to the presence of IgM antibodies in whole serum sample. Results in Table 4 reflect the presence of IgG antibodies crossreactive with both GM3 and GM4, which is similar to that seem with whole serum. These results are similar to those reported by Naiki et al. (119), but again reflects the highly heterogenous response among rabbits to these antigens.

**Immunofluorescent Staining**

An indirect immunofluorescent technique, using a rabbit antiserum to GM1, and a FITC-labeled F(ab')2 fragment of goat anti-rabbit IgG F(ab')2, was used to demonstrate antigenically exposed GM1 or GM1-like molecules on the surface of human lymphocytes. The distribution of cell surface fluorescent staining had one of three patterns: a uniform rim, large patches, or a granular pattern (Plate II). Staining also varied in intensity and was without significance relative to the cell source. Prior fixation of the cells with 4% formalin in SBSS resulted in a more intense and uniform pattern of staining. Similar observations have been made in the rat (169), in mice (184) and in man (165).

The percentage of lymphocytes which exhibit positive staining with antisera to GM1 varies with both the cell source and the anti-GM1 serum
used (Table 5). Adult peripheral blood (APB) lymphocytes and thymocytes demonstrate no difference in their expression of a $G_{M1}$-like antigenic determinant; 20.3 ± 9.3% and 20.0 ± 5.0% respectively when 14P Ig-UC anti-$G_{M1}$ serum was used. The percentage of positive staining APB lymphocytes decreases from 20.3 ± 9.3% to approximately 10% (range 7.7 ± 1.9 to 11.6 ± 2.6) when affinity purified antibodies to $G_{M1}$ are used in comparison to the whole immunoglobulin fraction of an anti-$G_{M1}$ serum, 14P Ig-UC (Table 5). This difference may reflect the presence of naturally occurring antibodies to human lymphocyte antigens in rabbit serum which can not be retained by a $G_{M1}$-affinity column. This latter contention is supported by the percentage of APB lymphocyte and thymocytes, 7.7 ± 2.7% and 5.0 ± 4.0% respectively, nonspecifically stained with normal preimmune serum. Individual differences in the surface expression of a $G_{M1}$-like antigenic determinant by APB lymphocytes is considered here to be normal and has been similarly noted by others (1,165).

There is an apparent differential expression of $G_{M1}$-like antigenic determinants on the surface of APB lymphocytes and lymphocytes derived from fetal cord blood (FCB) (Table 5). Depending on the affinity purified antiserum used 29.0 ± 12.8% (15P-AP) or 41.9 ± 27.3% (17P$_1$-AP) FCB lymphocytes stained as compared to 11.0 ± 2.6% (15-AP) and 7.7 ± 1.9 (17P$_1$-AP) APB lymphocyte staining may not only reflect individual differences but also that of glycosyltransferase activity for the synthesis of more complex gangliosides from $G_{M1}$ or a $G_{M1}$-like precursor molecule.
Subpopulations of lymphocytes taken from adult peripheral blood could be identified on the basis of staining with anti-\( \text{G}_{\text{M1}} \) sera (Table 5). Incubation of PBMCs on a nylon wool column results in a retention of B cells and monocytes while concentrating T cells and null cells in the eluate. The \( 35.3 \pm 8.1\% \text{G}_{\text{M1}}^+ \) nylon wool adherent cells represent B cells and correspond to the \( 37.5 \pm 3.7\% \text{G}_{\text{M1}}^+ \text{slg}^+ \) B cells found in unfractionated samples (Table 5). The nonadherent cell population contains \( 20.0 \pm 1.4\% \text{G}_{\text{M1}}^+ \) cells, whereas \( 13.2\% \pm 3.8\% \) of E rosette positive T cells obtained from unfractionated PBMC preparations stain. The difference may be due to B cell contamination and/or the presence of \( \text{G}_{\text{M1}}^+ \) null cells. A small population, approximately 3.0%, of APB lymphocytes are removed by plastic adherence, but further identification of these cells has not been made. Among the nonlymphocyte cell types there was no significant staining of erythrocytes or platelets; only a small population, approximately 10 to 15%, of the monocytes appeared to be \( \text{G}_{\text{M1}}^+ \) whereas all PMNs examined stained positively. This latter finding corresponds to the results of Ackerman et al. (1,2) using cholera toxin labeled anti-cholera toxin staining techniques and to the biochemical analysis by Hacher et al. (103) who reported that \( \text{G}_{\text{M1}} \) is the principal ganglioside in the membrane of human PMNs.

**Enzyme Pretreatment**

A series of experiments using different protocols for cell pretreatment with enzymes were designed to assess both nonspecific staining and to determine the degree of steric hindrance in the
antigenic expression of GM1-like molecules. In order to minimize nonspecific staining due to the binding of Fc receptors by antibody aggregates all antiserum was periodically ultracentrifuged at 100,000 x g for 60 minutes. In the studies below, Fc receptors for IgM molecules were removed by the minimal concentrations of trypsin (1.0 mg/ml), protease (1mg/ml) and VCN (500 u/ml) used, while the Fc receptors for IgG are only susceptible to the action of protease (112,134,162). Results using 13P, and 14P (Table 5) indicate that there is no significant alteration in the percent of GM1+ APB lymphocytes or thymocytes following proteolytic enzyme pretreatment as compared to untreated cells. Whereas, a comparison of untreated (Table 5) and enzyme pretreated (Figure 2) APB-lymphocytes indicates that 14P Ig does recognize a membrane component susceptible to removal by trypsin and protease. This component is thought to be a crossreactive glycoprotein rather than a Fc receptor or possibly ganglioside bound to a glycoprotein. Nonspecific staining by a preimmune serum (14 PIB Ig) and a normal rabbit serum (NRS) is greatly reduced following removal of trypsin or protease sensitive membrane components (Table 6, Figure 2). Addition of GM1 or GT1 to cells (Table 8) does not increase the percentage of cells staining with NRS as compared to untreated cells.

In comparison to untreated and proteolytic enzyme pretreated cells, VCN pretreatment of cells results in a significant increase in both the percentage of GM1+ cells and in staining intensity. (Tables 6, 7 and 8, Figures 2, 3 and 4, and Plate II) A more uniform surface staining was seen after VCN pretreatment as compared to the predominantly granular staining pattern seen on untreated cells (Plate II). The ability of
VCN, but not proteolytic enzymes, to unmask cryptic G\(_{M1}\) or G\(_{M1}\)-like antigenic determinants is demonstrated by two facts. At antibody protein concentrations of 20 \(\mu\)g/tube, or less, there is no significant increase in the percentage of G\(_{M1}^+\) cells following trypsin or protease pretreatment (9.2±5.2\%) as compared to untreated cells (8.8±1.8\%). This compares to a marked increase in G\(_{M1}^+\) cells, ranging from 16.0\% to 100\% depending on the antiserum used, following VCN pretreatment (Tables 6 and 7). The exposure of "cryptic" antigenic determinants by VCN is seen with cells from all sources, and includes platelets, monocytes, PMNs as well as lymphocytes. In only a few instances was staining of erythrocytes observed following VCN pretreatment. The variability in results using different antisera and in experiments using the same antiserum results in no determination of a differential cellular expression of these "cryptic" antigens. Such variability in VCN activity was reported by Schulof et al. (162) who reported that a VCN mediated decrease of T cell Fc receptors for IgM varied from 59.6\% to 100\%, and attributed this to donor variability. The variation seen in these experiments result from three major factors: donor variation, differential expression of VCN exposed antigens, and incomplete removal of sialic acid residues by VCN resulting in some antigenic determinants still unable to bind with antibodies to G\(_{M1}\).

The chemical nature of the "cryptic" antigen exposed by VCN was studied in a series of experiments represented in Figures 2, 3, and 4. Treatment of VCN pretreated cells with increasing concentrations of trypsin and protease did not significantly decrease the percentage of G\(_{M1}^+\) cells as compared to VCN pretreated cells without post treatment.
There is no significant difference in the expression of these "cryptic" antigens if the cells are first treated with trypsin and then VCN or in reverse order. Therefore the "cryptic" antigen(s) exposed by VCN are not sensitive to trypsin or protease digestion. Also the removal of trypsin sensitive components from the membrane does not alter the subsequent VCN exposure of the "cryptic" antigen. The carbohydrate nature of the antigenic determinants exposed by VCN is seen in Figure 4. Whereas pretreatment of cells with sodium m-periodate (NaIO₄), B-galactosidase or B-glucosidase does not significantly alter the percent staining of non-VCN treated cells, they do appear to decrease antibody binding to cells pretreated with VCN. This corresponds to the hapten inhibition experiments by Rogentine and Plocinik (149) and Parish et al. (131) who reported that galactose and its dimers of galactose can inhibit antibody binding to VCN exposed antigens. These are also considered to be the principal antigenic determinants recognized by antiserum to G₄M₁ (119). Nonspecific staining by 14F1B and NRS can be minimized by using low protein concentrations. The antigenic determinants recognized by these nonimmune sera are partially sensitive to proteolytic enzyme pretreatment.

**Ganglioside Pretreatment**

There is significant increase in the percentage of APB-lymphocytes stained following their uptake and membrane incorporation by these cells of exogenous G₄M₁ (Table 8). There is little increase in the staining intensity and the appearance is granular rather than a uniform rim of
stain (Plate II). Increases in staining percentage following GM1 pretreatment are also noted for: erythrocytes, platelets, and monocytes. The specificity of this increase in staining is demonstrated by two observations. In the first case there is no increase above control levels for cells pretreated with GT1 rather than GM1 and stained with anti-GM1 serum, and secondly there is no increase in staining in cells pretreated with either GM1 or GT1, and stained with NRS. The staining of GM1 pretreated cells with 15P and 17P1, is less than 30% as compared to greater than 75% of similarly pretreated cells stained with 14P−Ig or 16P4. This difference reflects the high antibody titer and possibly a strong avidity of the latter antisera. This difference is also seen with those cells pretreated with VCN. The increase in staining percentage following membrane incorporation of GM1 parallels that seen following VCN pretreatment. This observation adds further support to the conclusion that VCN "cryptic" antigens are, in part, GM1 or GM1-like in their antigenic expression.

Capping

At 0−4°C, the indirect immunofluorescent staining of GM1 on the surface of APB−lymphocytes result in either a uniform rim pattern or large patches of staining. Incubation of the cells at room temperature or at 37°C lead to a rapid rearrangement of GM1 antigenic determinants into distinct patches and caps (Plate III). The rate of cap formation with antisera to GM1 was rapid and progresses over a 20 minute period (Table 9). There is an inverse relationship between the percentage of
cells stained and the number of capped cells. The use of a second antibody to crosslink anti-\(G_{M1}\) bound to the surface and induce capping was found not to be necessary, in that anti-\(G_{M1}\) alone can crosslink \(G_{M1}\)-like determinants and induce its membrane redistribution into caps. This is in accordance with findings with rat thymocytes (169).

The general progression of cap formation follows that noted for the capping of membrane bound immunoglobulin (mIg) (160). Small irregular clusters, yielding a granular appearance, are the first indications of the rearrangement of \(G_{M1}\) in the membrane and often times occurs when incubation temperatures are not maintained at \(4^\circ\text{C}\) or less. The small clusters coalesce into large patches which in turn form the cap. With increasing time, the cap is removed by endocytosis or shedding. The granular and patchy appearance often times seen in noncapping studies can be attributed to the rapid rate of redistribution of these determinants when the temperature exceeded \(4^\circ\text{C}\) at some point prior to fixation. Caps are seen, (Plate III), as being primarily small and no larger than 25% of the cells surface. The location of the cap varies relative to the position of the nuclear hof. Associated with cap formation is the formation of cytoplasmic blebs and organelle filled uropods. Bleb formation occurs opposite that of the cap, while the cap overlies the tip of the uropod (Plate IV). The organelle rich uropod was often times seen to constrict and give rise to a membrane bound vesicle containing the capped \(G_{M1}\) determinants. Cap formation does not always give rise to such changes in cell morphology. The formation of uropods in association with \(G_{M1}\) cap formation has also been described by
Sela et al. (169). Schreiner and Uranue (160) have also described a similar association with the capping of mIg and concluded that it was related to an induction of cell motility. A less frequent morphological change than that of uropod formation was an equatorial constriction of the cell with clusters of staining seen to line up along the equatorial plane. Capping of Gm1 was also seen on the surface of monocytes, PMNs, platelets and thymocytes, but was not quantitate for this study.

Affinity purified anti-Gm1 serum was also capable of inducing the capping of cryptic Gm1-like antigenic determinants exposed by VCN as well as exogenous Gm1 taken up and incorporated into the cell membrane (Plate IV). The rate of capping parallels that seen with untreated cells (Table 9), and the capping of exogenous Gm1 does not require a second antibody. Capping of both VCN and Gm1 pretreated cells induces uropod formation at the site of cap formation. Fewer caps are associated with uropods in Gm1 as compared to VCN pretreated cells, but in each case the uropods contained organelles. In some cases, small clusters of membrane staining were found on VCN pretreated cells with uropod associated caps. The constriction of these uropods (Plate IV), as seen with untreated cells, resulted in the formation of membrane bound vesicles, and can be considered as the mode for 'cap shedding.' Endocytosis, seen as cytoplasmic staining, was noted as occurring more in conjunction with Gm1 pretreated cells than those cells untreated or pretreated with VCN.
Capping Inhibition

Table 10 is representative of results from experiments designed to characterize the effects of pharmacological reagents on the capping of GM1, and to compare these results to those seen with cells pretreated with VCN or exogenous GM1. Capping of intrinsic GM1, but not its aggregation, is an active process requiring an intact system for generation of ATP. Disruption of oxidative phosphorylation by sodium azide (0.01M) and 2,4-DNP(5 x 10⁻⁴M), and the inhibition of glycolysis by 2-deoxy-D-glucose (0.05M) and sodium fluoride (0.1M) leads to a loss of metabolic energy resulting in an inhibition of GM1 cap formation. In contrast to a dependency on ATP synthesis, capping of GM1 is unaffected by an inhibition of protein synthesis by cycloheximide. Disruption of cytoplasmic microfilaments by cytochalasin B results in a variable inhibition of GM1 capping. The effect of cytochalasin B ranges from total inhibition to no inhibition of GM1 cap formation. Similar results have been reported in relation to capping of membrane immunoglobulin (reviewed by Schreiner and Unanue 160). A disorganization of microtubules within the cytoplasm by colchicine yields variable results, but it tends to enhance the formation of GM1 caps rather than inhibit their formation. When combined at a 1:1 ratio, cytochalasin B and colchicine act synergistically to inhibit the formation of GM1 caps. This joint disruption of cytoplasmic microfilaments and microtubules is also accomplished by lidocaine (137), and again leads to an inhibition of the membrane rearrangement of GM1 into caps.
The aggregation of VCN exposed antigenic determinants by antibodies to $G_{M1}$ is independent of an intact cytoskeletal system as well as a need for energy or protein synthesis. Similarly, various pharmacological reagents could not inhibit antibodies to $G_{M1}$ from inducing a passive rearrangement of exogenous $G_{M1}$ incorporated into the plasmalemma. In contrast, the capping of these membrane structures by anti-$G_{M1}$ is energy dependent. The ability of cycloheximide to inhibit exogenous $G_{M1}$ and VCN exposed $G_{M1}$ or $G_{M1}$-like determinants can not be explained. When both microfilaments and microtubules are disrupted by lidocaine or a 1:1 colchicine : cytochalasin B solution, there is an inhibition of capping to the exogenous $G_{M1}$ and VCN exposed determinants. In contrast to this latter finding, capping in both cases is enhanced by colchicine. Concomitant with enhanced capping there is an increase in the number of cells forming uropods at the site of cap formation in the presence of colchicine. The presence of cytochalasin B during capping has a variable impact on capping of both VCN exposed antigens and exogenous $G_{M1}$ by antibodies to $G_{M1}$. As in the case with intrinsic $G_{M1}$, the effect of cytochalasin B varies in its extent of inhibition.

**Cocapping of $G_{M1}$ with Known Membrane Components**

Cocapping experiments (Tables 11, 12, and 13) were designed to determine if $G_{M1}$ has a physical association with three known membrane proteins: membrane bound immunoglobulin (mIg), $B_2$-microglobulin and "Ia" antigens. The results of studies using B cells obtained from PBMC's and nylon wool adherence show that $G_{M1}$ cocaps with mIg in 100% of the
containing cells. The molecular association between GM1 and mIg is most likely mediated by sialic acid residues. This conclusion is derived from results showing a complete loss of cocapping following VCN pretreatment of the cells (Table 11). VCN pretreatment does not inhibit the formation of either GM1 or mIg caps. There is no apparent association between GM1 and B2-microglobulin as seen by the inability of these two molecules to comigrate into a single cap (Table 12). Exposure of additional antigenic determinants by VCN, and recognized by affinity purified antibodies to GM1, does not result in an association of these determinants with B2-microglobulin. There is an apparent physical association between B cell Ia-like antigens and GM1 which is resistant to disruption by VCN (Table 13). This physical association, unlike that with mIg, varies in that not all Ia caps contain GM1 and visa versa. It is also apparent that the antigenic determinants revealed by VCN, and capable of being recognized by antibodies to GM1, are only partially associated with Ia antigens, which is indicated by a decrease in percentage of cells containing and cocapping as compared to untreated cells. The association between the GM1-like and Ia antigens may also be due to shared antigenic determinants recognized by both antisera; a possibility indicated by the results where monoclonal antibodies to Ia sterically inhibit the binding of antibodies to GM1 from binding to cell but not visa versa. A similar inhibition is not seen following the pretreatment of cells with VCN.
**Immunoprecipitation**

To determine if the antigenic determinants recognized by antibodies to G\(_{M1}\) are located on different cell surface glycoconjugates, a series of immunoprecipitation experiments were conducted using tritiated and radioiodinated membrane extracts. PBMC's were pretreated with both VCN and galactose oxidase (NGO) and subsequently labeled with tritiated sodium borohydride (NaB\(_3\)H\(_4\)). Immunoprecipitation of solubilized extracts of labeled membrane with antibodies to G\(_{M1}\) (16P\(_4\) IgG) and SPA resulted in two low molecular weight peaks being found on 7.5% SDS-PAGE gels (Figure 5). One is a broad peak with an approximate molecular weight of (11,000 daltons), while the second is a sharp peak having a molecular weight of (7,000 daltons). There was no significant immunoprecipitable of a membrane component with 16P\(_4\) IgG following NaB\(_3\)H\(_4\) labeling of cells pretreated with PBS, galactose oxidase or NaI0\(_4\). The lack of one or more glycoproteins in the immunoprecipitate is in contrast to the results of Tonegawa and Hakomori (190) who demonstrated that antiserum to G\(_{M1}\) could coprecipitate up to four glycoproteins in addition to G\(_{M1}\). The difference lies in their use of murine fibroblasts and my use of human PBMCs. The relative position of these two peaks corresponds to that of Tonegawa and Hakomori (190) using rabbit antiserum to G\(_{M1}\), and also corresponds to the results of Critchley et al. (33) who used cholera toxin–anti–cholera toxin. Further characterization of 16P\(_4\) IgG's ability to recognize and precipitate membrane associated G\(_{M1}\) is seen in the results, (Figure 5),
of experiments where cells were first incubated with $^{3}\text{H-}G\text{M}_1$ and then processed in a manner identical to that with NaB$^{3}\text{H}_4$ labeled cells. The resulting single peak corresponds to a molecular weight of (12,000 daltons). This value is higher than that with immunoprecipitates of NGO pretreated and NaB$^{3}\text{H}_4$ labeled cell extracts, but all three are still within the range of molecular weights for ganglioside micelles. A single peak results from the immunoprecipitation of NGO-NaB$^{3}\text{H}_4$ labeled extracts (Figure 5) with NRS IgG. This may represent a general property of gangliosides to bind nonspecifically to IgG molecules (Chambers et al. submitted for publication) and/or the presence of anti-ganglioside antibodies in normal rabbit serum. The latter case seems unlikely in that such antibodies would be of an IgM class rather than IgG molecules. This nonspecific precipitation results in less of a drag down as compared with 16P$_4$ IgG; even though, 17% greater cpms were added to the gel containing the NRS precipitate.

The inability of antibodies to $G\text{M}_1$ to crossreact with and immunoprecipitate a membrane glycoprotein was further studied using membrane extracts of PBMCs labeled by lactoperoxidase - H$_2$O$_2$ catalyzed radioiodination ($^{125}\text{I}$) (Figure 6). The high molecular weight peaks, those greater than 30,000 daltons, are considered to be artifacts resulting from nonspecific binding to SPA and/or NRS IgG. Near identical peaks, artifacts, are seen whether the cell extracts are pretreated with PBS, NRS IgG or anti-$G\text{M}_1$ IgG and subsequently precipitated with SPA. There is a significant increase in the cpms
found at the front of the 16P4 IgG in comparison to the PBS and NRS IgG gels; even though 20% fewer counts were added to the anti-C_M1 gel. That this gel front peak may indeed represent 125I-C_M1 comes from studies where 3H-C_M1 was directly added to a gel and the resulting peak was found at the gel front. This observation extends that of Schlager (157) who reported that various membrane lipids could be radioiodinated in addition to the exposed tyrosine residues of membrane proteins.
Antibodies to the ganglioside G\(_{M1}\) can identify a differentially expressed antigenic determinant on subpopulations of human T and B lymphocytes. The properties and behavior of this antigen, or antigenic determinant, in the plasma membrane of lymphocytes are those of a glycolipid. This antigen, or antigenic determinant, is resistant to the enzyme activity of both trypsin and protease; whereas, its expression is increased following neuraminidase pretreatment of cells. The antigenic determinant can be radiolabeled with tritiated sodium borohydride and immunoprecipitated to yield one or more molecules with a molecular weight of less than 12,000 daltons. The molecule(s) carrying this antigenic determinant can undergo rapid antibody induced rearrangement in the plane of the membrane to yield a cap. The antigen is not associated with B\(_2\)-microglobulin but is associated with both membrane bound immunoglobulin and an Ia-like antigenic molecule. These associations are disrupted by neuraminidase and therefore they are most likely mediated by sialic acid residues.

For the sake of simplicity, I will refer to the membrane antigen recognized by the antibodies to G\(_{M1}\) used in this study as the G\(_{M1}\)-like antigenic determinant. This determinant is considered to be a ganglioside and its biochemical properties will be discussed at a later point in this section. Data expressed on Tables 5 and 6 indicate that the G\(_{M1}\)-like antigenic determinant is expressed on the surface of
the \( \text{G}_\text{M}_{1} \)-like antigenic determinant is expressed on the surface of lymphocytes from different tissue, however, the percentage of positive cells varies with the antiserum as well as with the tissue source of lymphocytes. The fact that the use of different antisera can yield differing results and conclusions is particularly evident when comparing the results of untreated cells with those of cells pretreated with VCN or \( \text{G}_\text{M}_{1} \) (Table 8). These variations are, in part, due to differences in antibody titer, avidity and specificity. Such differences are common among antisera to gangliosides (106,140) and will be discussed further later in this section.

Individual differences in the expression of the \( \text{G}_\text{M}_{1} \)-like antigenic determinant are seen when the same antiserum is used. This is expressed by the standard deviation, and can be seen to be much greater with FCB-lymphocytes than with thymocytes or APB-lymphocytes. Similar variations occur with monoclonal antibodies to human T (48,143) and B (21,38). Individual differences in binding antibodies to \( \text{G}_\text{M}_{1} \) has been demonstrated in mice (183,184) and humans (165). Ackerman et al. (1) reported variation in cholera toxin binding to cells from different individuals.

Classically, human T and B lymphocytes have been distinguished on the basis of their expression of membrane bound immunoglobulin (mIg) and receptors for sheep erythrocytes, immunoglobulin (Fc) and complement components (C3) (26). Human T cells (144) and B cells (21,38,116,181) have been divided additionally into subpopulations based on their binding of monoclonal antibodies. The antigenic determinants recognized by these various antibodies are glycoprotein in nature (38,116,138).
Gangliosides and other glycosphingolipids (GSLs) are normal constituents of lymphocyte membranes (103), but only recently have these molecules been described as antigenic determinants on lymphocyte plasma membranes (165). G_m-like antigenic determinants, recognized by antiserum to G_m or asialo-G_m, are expressed on murine T and B cells (184), differentiating murine thymocytes (67,183), differenting T cells in the rat (7) and on natural killer cells from various species (83,210), including man (Herberman-personal communication).

Schwarting and Marcus (165) using antisera to various GSLs, including G_m and asialo-G_m were able to identify various subpopulations of human T and B cells. Results here confirm these observations as they pertain to staining with antibodies to G_m. Approximately 13% of the E-rosette positive APB-T cells and 37.5% of the mIg positive APB-B cells are seen to express the G_m-like antigenic determinant (Table 5). Of particular interest is the fact that Schwarting and Marcus (Table 3 in 165) did not find a significant difference in the percentage of cells staining with antibodies to G_m or asialo-G_m. Approximately 35% of the cells enriched by nylon wool adherence stain, which is the same result seen with double staining techniques. In contrast, of the enriched non-adherent T cells and null cells only 20% were noted as expressing the G_m-like antigenic determinant. In comparison with the 13% of E-rosette positive T cells staining, this additional 7% is considered here to be due to null cell staining. A major null cell population are the large granular lymphocytes, which represent 5-10% of the APB-lymocytes and are positively stained by antisera to asialo-G_m (Herberman-personal
communication). It was consistently observed that approximately 3% of positive staining lymphocytes were lost by culturing for 2 hours in order to remove monocytes. This finding may either represent a very labile cell population susceptible to culturing or a plastic adherent population of lymphocytes.

The ability of the GM_1-like antigenic determinant to serve as adifferentiation antigen for human lymphocytes is indicated by the two-fold decrease in positive staining lymphocytes from adult peripheral blood as compared to FCB-lymphocytes (Table 5). Ackerman et al. (2) reported increases in membrane GM_1 in differentiating bone marrow cells of the myeloid, erythroid and monocyte series. Neutrophils, as well as monocytes, obtained from either fetal cord or adult peripheral blood demonstrated staining, but their numbers were not quantitated in this study. In comparison, Tsuru et al. (191) have reported a decrease in T cell binding of cholera toxin, indicating a decrease in membrane GM_1, during aging. As yet, the data presented here can not rule out the fact that the GM_1-like antigenic determinant may be a differentiation antigen for more than one cell type. In humans, various glycoproteins have been reported to be differentiation antigens for both T (144) and B (116) cells. GM_1-like antigenic determinants are considered to be differentiation antigens for murine thymocytes and T cells (67,183,184) and rat T cells (7). Similar determinants are being considered as differentiation markers for human T cells based on their expression on non-B cell-ALL cells (121,166). The differential expression of the GM_1-like antigenic determinant may be the result of activation of multiglycosyl transferase systems responsible for glycolipid synthesis.
during development and maturation. This is the proposed mechanism for the conversion of i antigen to I antigen on erythrocytes with aging (ref. in 202), which is opposite to what is seen with I-i antigen on human lymphocytes (139).

Kabat (82) has stated that the antigenic determinant which is immunodominant on a short carbohydrate chain is the terminal, nonreducing, carbohydrate of the chain. He also reported (83) that antibodies to carbohydrate chains primarily bind 2 to 6 sugar residues and that these antibodies are "directed toward sequential rather than conformational determinants". The terminal galactose (Gal) and the penultimate N-acetyl-galactosamine (GalNAc) residues are, therefore, considered to be the immunodominant determinants on G\textsubscript{M1} (Table 1, Figure 1). The results seen in Tables 2, 3, and 4, as well as several reports (92,106,119), support this conclusion. Both G\textsubscript{M1} and G\textsubscript{D1b} have the same unsubstituted Gal(B1-3)GalNAc terminal residues; whereas, the terminal Gal is modified by one or more sialic acid residues in the case of G\textsubscript{Dla} and G\textsubscript{T1}. Subpopulations of antibodies in anti-ganglioside serum are also capable of reacting with just the terminal nonreducing residue (154,212), while others recognize and bind to an internal sequence of residues in the carbohydrate chain (82,106). The ability of antibodies to G\textsubscript{M1} to recognize and bind just to galactose has been reported by Saha and Ghosh (154) and is seen here (Tables 2, 3, and 4) in their ability to bind galactocerebroside. The ability of G\textsubscript{M1} reactive antibodies to bind to an internal sequence of residues in the carbohydrate chain is demonstrated here by the reactivity of different sera with G\textsubscript{M2}, G\textsubscript{M3} and G\textsubscript{M4} (Tables 2, 3, and 4). Reactivity of these various antisera with
GM2, GM3, GD1a and GT1 indicates that the internal sialic acid residue is also an important immunodeterminant recognized by antibodies to GM1. Antibodies to nonterminal sugar residues of globoside (163) and the I and i antigens (21) have been described in certain clinical conditions.

The presence of antibody subpopulations in anti-GM1 sera results in a wide range of sera reactivity and specificity. Marcus and coworkers (106, 119) have shown that variations in these subpopulations vary among different animals as well as different bleeds of the same animal. IgM and IgG antibodies of the same serum can differ widely with respect to antigen specificity (105); with the IgM molecules being considered to be the least specific (119). Results shown on Tables 2, 3, and 4 depict a decrease in non-GM1 ganglioside binding by IgG antibodies as compared to the whole serum. There is also a greater antigen specificity of the IgG fraction of the whole serum as compared with affinity purified antibodies. This difference is attributed to the presence of IgM antibodies in whole serum and affinity purified preparations.

The ability of the antisera used in these studies to bind to membrane GM1 is seen in the results of staining (Table 8) and immunoprecipitation (Figure 5) of membrane incorporated exogenous GM1 and 3H-GM1 respectively. The principal antigenic determinant recognized by antibodies to GM1 is the terminal Gal(Bl-3)GalNAc. This same carbohydrate sequence is found on membrane glycoproteins (82, 91, 142) as well as on asialo-GM1 (Table 1). Additional carbohydrate associated epitopes recognized by these antibodies are expected to be common to various membrane glycoconjugates. Yet, the GM1-like antigenic determinant studied here on human lymphocytes lacks the characteristics
of a protein. It is resistant to both trypsin and protease, and only slightly affected by B-glucosidase, B-galactosidase or periodate oxidation. Immunoprecipitation of membrane lysates from PBMCs pretreated with VCN-galactose oxidase and labeled with NaB$_3$H$_4$ results in two peaks with molecular weight of approximately 10,000 and 7,000. Immunoprecipitation of $^3$H-$G_{M1}$ incorporated into cell membranes results in a single peak with a molecular weight of 11,000 (Figure 5). These three molecular weights are consistent with the molecular weight of $G_{M1}$ micelles. Immunoprecipitation of $^{125}$I labeled PBMC membrane lysates (Figure 6) again results in no significant peak before the gel front. These results conflict with those of Tonegawa and Hakomori (190), who reported that up to four "ganglioproteins" were precipitated, in addition to $G_{M1}$, with antiserum specific for $G_{M1}$. This discrepancy results from the fact that different cells (murine 3T3 fibroblasts), and whole antiserum (not the IgG fraction used here) were used in their study.

Biochemical studies have shown that $G_{M3}$ (hematoside Table 1) is the primary ganglioside of human lymphocytes and thymocytes (74,182). Recently, these results have been extended by Macher et al. (103) who proposed that there are three major gangliosides in addition to $G_{M1}$ and $G_{M3}$ which are found in human lymphocyte membranes. Their structures are as follows:

\[(1)\]

\[\text{NANA(x2-)} \text{GlcNAc(B1-3)Gal(B1-4)Glc(B1-1)Cer}\]
In addition to this latter complex ganglioside, Pruzanski and Shumak (139) have shown that the complex ganglioside I-i antigens (202) are expressed on the surface of human lymphocytes. The three carbohydrate sequences described by Hacher et al. (103) and those for I-i antigens (27) resemble paragloboside and sialosylparagloboside (164):

**Paragloboside**

\[ \text{Gal(Bl-4)GlcNAc(Bl-3)Gal(Bl-4)Glc(Bl-1)Cer:} \]

**Sialosylparagloboside**

\[ \text{NANA(x2-3)Gal(Bl-4)GlcNAc(Bl-3)Gal(Bl-4)Glc(Bl-1)Cer:} \]

To date, there are no reports of antiserum to GM1 crossreacting with these latter glycosphingolipids, nor have I tested them. Using antisera to paragloboside, Schwarting and Marcus (165) have reported that 15-20% of APB-lymphocytes, 50-60% B cells and 5-10% T cell stain positively. It is possible that their antisera recognize these newer and more complex gangliosides. Of interest here, is the report that replacement of GalNAc with GlcNAc does not alter the ability of GM1 to bind cholera toxin (53).
The binding of anti-\(G_M^1\) antibodies to the surface of human lymphocytes does not correlate to the type or the amount of ganglioside in the cell membrane. Studies using cholera toxin - anti-cholera toxin have shown that all human lymphocytes contain \(G_M^1\) in their plasma membrane \((1,146,147)\). The inaccessibility of \(G_M^1\)-like antigenic determinants for antibody binding may result from a steric hindrance or masking by other membrane components. Results shown in Table 6 demonstrate that the removal of membrane constituents with trypsin and/or protease did not significantly expose additional antigenic determinants of this type on the surface of human lymphocytes. Similar results have been reported using human \((165)\) and rat \((169)\) lymphocytes, but are in contrast to those of Stein et al. \((183)\) using murine lymphocytes. Whereas proteolytic enzymes do not expose \(G_M^1\)-like antigenic determinants they do expose \(G_M^3\) \((discussed\ in\ 105)\) and neutral glycolipids \((164)\).

The ability of VCN pretreatment of human lymphocytes to expose or unmask "cryptic" \(G_M^1\)-like antigenic determinants is in contrast to the activity of proteolytic enzymes \((Tables\ 6,\ 7,\ and\ 8,\ Figure\ 2)\). VCN functions to remove sialic acid residues from the carbohydrate chains of membrane glycoconjugates, and exposes additional galactose residues at the nonreducing terminus of these chains \((176)\). The carbohydrate nature of the exposed determinants is indicated by their susceptibility to \(\beta\)-glycosidases, and the presence of a sialic acid residue is seen in their susceptibility to sodium periodate oxidation \((Figure\ 4)\). The determinants are not associated with a trypsin sensitive protein, but
may in part be associated with a protease sensitive protein (Figures 2 and 3). Labeling of these unmasked determinants with galactose oxidase-\(\text{NaB}_{3}\text{H}_{4}\) does not yield a glycoprotein following immunoprecipitation with IgG antibodies to \(\text{G}_{\text{M}}\)\(_1\), but does result in two low molecular weight peaks corresponding to a \(\text{G}_{\text{M}}\)\(_1\)-like molecule (Figure 5). These findings, which indicate that VCN exposes antigenic determinants resembling \(\text{G}_{\text{M}}\)\(_1\), are supported by results of studies which showed that VCN pretreatment of cells resulted in an increase in binding of: cholera toxin (1,69,70,76), peanut agglutinin (164,176) and antibodies to asialo-\(\text{G}_{\text{M}}\)\(_1\) (164,188) and \(\text{G}_{\text{M}}\)\(_1\) (109). In addition, VCN pretreatment of human lymphocytes exposes additional gangliosides with \(\text{I}-\text{i}\) antigenic activity (51). The biochemical nature of the VCN-sensitive substrate is seen here to be partially protease sensitive (Figure 2), but no conclusion can be made as to whether the substrate is a complex ganglioside or not. The variability in staining of VCN pretreated cells with anti-\(\text{G}_{\text{M}}\)\(_1\) sera is considered to reflect the amount of crossreacting IgM molecules in the serum. A second explanation would be that a small population of lymphocytes is incapable of reacting with VCN and therefore would lack cryptic antigenic determinants capable of reacting with antibodies to \(\text{G}_{\text{M}}\)\(_1\). There is too great a variability in staining percentage of VCN pretreated cells when different antisera are used (Tables 6,7 and 8). Therefore this variability is not considered by me to be due to a restriction in the cellular expression of the VCN unmasked determinants.
Gangliosides function in membrane recognition of several biologically active agents (reviewed in 23) and subsequently they mediate the transduction of this signal from the surface into the cytoplasm (18,66,179). Changes in membrane permeability may induce signal transduction. Such changes have been found with ligand induced clustering of gangliosides in artificial membranes (42,114), and have been proposed to occur following a ligand induced formation of lipid domains in human lymphocyte membranes (36). Signal transduction may be secondary to the interaction of ligand-ganglioside complex with another membrane protein. This interaction would result from a lateral redistribution of complex in the plane of the membrane (12,197). Antibodies to DNP-GM1 (179) and to GM1 (169) bind to rat thymocyte surfaces and induce mitogenesis. These authors considered that the redistribution of the antibody-ganglioside complex into a cap results in the transduction of a mitogenic signal across the membrane. In support of this conclusion, Curtain (35) has shown that both an anti-GM1 GM1-complex and cAMP are located in the same cap in human lymphocyte membranes.

The lateral mobility of a ganglioside-ligand complex in the plane of the membrane is therefore significant as a model in the understanding of the interactions between membranes and the cytoplasm. Using mammalian lymphocytes, ligand induced redistribution of membrane GM1 has been studied using cholera toxin alone (29,30) or in combination with antibodies to cholera toxin (1,70,77,147) and antibodies to GM1 (169,183). In addition, Spiegel et al. (179) and Sedlacek et al. (168),
have studied the lateral mobility of exogenous G\textsubscript{M1} once incorporated into the cell's membrane. This portion of my dissertation was designed to look at the features of antibody induced redistribution of the G\textsubscript{M1}-like antigenic determinant and exogenous G\textsubscript{M1} in the membrane of human lymphocytes, and to determine the role that energy metabolism and cytoskeletal elements play in it.

Cross linking of the G\textsubscript{M1}-like antigenic determinant and membrane incorporated exogenous G\textsubscript{M1} results in their rapid redistribution into patches and caps (Table 9, Plate III). Schroit and Pagano (161) reviewed the fact that a monovalent, membrane associated, hapten could not be redistributed by a bivalent antibody. Gangliosides are haptens (105), but also are antigenically multivalent as noted by their ability to elicit antibodies with numerous specificities (Tables 2, 3, and 4). In the cell membrane gangliosides can also be considered to be multivalent due to their self-association, or aggregation (172). The formation of microaggregates of membrane incorporated exogenous G\textsubscript{M1} has been noted in artificial membranes (42), and would account for their ability to undergo an antibody induced redistribution in the lymphocyte membrane. These results are in apparent disaggreement with Nicolson's statement (124) that "there is an inverse relationship between the proximity of analogous surface components and capping efficiency", that is, one would expect a decrease in the ability of aggregates or self-associated molecules to undergo cap formation. Such an inverse relationship may be true for membrane glycoproteins but not for glycolipids as seen here and elsewhere (31, 169, 183). The induction of
membrane redistribution of both the $G_{M1}$-like antigenic determinant and membrane incorporated exogenous $G_{M1}$ requires a multivalent or divalent ligand; either cholera toxin (31,168) or antibodies as seen here and by Sela et al. (169). Results of preliminary experiments showed that these membrane determinants could be redistributed by $F(ab')_2$ but not Fab fragments of IgG antibodies to $G_{M1}$.

The antibody stimulated redistribution and cap formation of the $G_{M1}$-like antigenic determinant and membrane incorporated exogenous $G_{M1}$ is comparable to that of membrane bound immunoglobulin (mIg) (Table 14). Temperatures below 4°C increase membrane viscosity to where redistribution of these antigens is minimized, but at 4°C or higher there is an increase in granular staining and patch formation. Brady and Fishman (18) have reported that even at 4°C activation of adenyl cyclase can occur following cholera toxin binding to the cell surface. This is taken as meaning that the $G_{M1}$-ligand complex can undergo lateral mobility even at this low temperature. Part of the variation in results of this study, and others, could be due in part to not maintaining all experimental conditions at or below 4°C. I have also noticed, subjectively, that cap formation is slower at room temperature than at 37°C. This corresponds to the observation that "gangliosides undergo a phase transition between 25°C and 37°C and, therefore, lose much of their rigidifying effect" (175). The rapid rearrangement of the $G_{M1}$-like antigenic determinant and membrane incorporated exogenous $G_{M1}$ induced by heating at 37°C (Table 9) is in accordance with measurements of membrane $G_{M1}$ lateral mobility (40). In this latter study, it was
concluded that nearly 100% of a FITC-labelled exogenous GM1 was mobile in the plane of the membrane as compared to only 30% of the membrane protein. This would indicate that exogenous GM1, when incorporated into a cell membrane, does not behave as does the intrinsic GM1. This possibility is supported by the susceptibility of membrane incorporated GM1 to the action of neuraminidase in contrast to intrinsic GM1, (86,87). Yet I found both to behave in an identical manner, which is in accordance with the results of Moss et al. (113) who showed that cholera toxin resistant cells respond to the toxin following membrane incorporation of exogenous GM1.

Schreiner and Unanue (160) have reported that while both patching and cap formation of mIg is temperature dependent, only capping is dependent of cellular energy production. This was also seen with anti-GM1 induction of cap formation in each of the three systems studied (Table 10). The requirement for energy during GM1 cap formation has also been shown using antibodies to GM1 (169) and cholera toxin (31,147). As seen in this study, the dependency on energy may be related to a contraction of cytoskeletal elements leading to cap formation. This was proposed by Ostro et al. (129) for mIg cap formation. By increasing lipid fluidity there is a loss of cytoskeletal interaction with mIg which is exhibited by independency of energy (129). Similar results have been reported using an anti-TNP TNP-phosphotidylethanolamine model (161).

Lymphocyte mIg is not considered to be a transmembrane protein (129,200), and like gangliosides, it therefore is not directly
associated with cytoskeletal elements. Yet the binding of anti-Ig to lymphocytes is thought to initiate such an association (17,19,54). The initiating event appears to be an alteration in the continuous flow of membrane lipids (20), resulting in the formation of lipid domains (36). Gangloisides in artificial membranes cluster following the binding of lectins (42). Curtain et al. (36) have shown that the binding of various lectins to human lymphocytes results in clustering of GSLs. Ostro et al. (129) and Schroit and Pagano (161) have shown that such clustering will not lead to cap formation if membrane fluidity is increased by additional lipids being incorporated into the membrane. The addition and incorporation of $G_M^1$ to lymphocytes does not appear here to increase the membrane fluidity to the point of preventing cap formation (Plate III).

If the $G_M^1$-like antigenic determinant and membrane incorporated exogenous $G_M^1$ were only self-associated following cross-linking with antibodies to $G_M^1$ then one would not expect to see an inhibition or an enhancement of capping by agents which disrupt the cytoskeletal elements. The ability of these pharmacological agents to affect anti-$G_M^1$ induced capping is seen on Table 10 and has been reported during cholera toxin (31,147,168) and anti-$G_M^1$ (169) induced capping. The variability in the effect of these drugs was noted earlier, and is reflected in the results of these above authors. Following the initial anti-$G_M^1$ induced clustering, the $G_M^1$-like antigenic determinant and membrane incorporated exogenous $G_M^1$ associate with elements of the cytoskeleton. Association is most likely an indirect association
mediated by one or more transmembrane proteins and involving both microtubules and microfilaments. Disruption of only microtubules with colchicine actually enhances capping as well as cap associated uropods, which is similar to the results with mlg capping (160). In contrast, the disruption of microfilaments by cytochalasin B results in an inhibition, although variable, of anti-GM1 induction of cap formation. The affect of these drugs on GM1 cap formation is not always reversible as reported by Loor (100). Acting synergistically, colchicine and cytochalasin B totally disrupt the cytoskeleton elements which results in near total inhibition of capping. This is also supported by the results with lidocaine, a tertiary amine anesthetic, which disrupts cytoskeletal elements by displacing membrane bound Ca+2 (19,137). The contraction of the microfilaments requires both Ca+2 and energy (17) and would thus explain ability of the various inhibitors used here to inhibit cap formation. Contraction of the microfilaments is considered to be similar to the sliding filament theory for muscle contraction. To further extend the present findings studies to determine if actin and/or myosin are associated with anti-GM1 induced caps need to be made.

A direct association of GM1 with one or more membrane proteins, resulting in a single unit, has been proposed by Craig and Cuatrecasas (31) to explain the control of GM1 capping by cytoskeletal elements. Such an association would have functional implications associated with: signal transduction (11,12,179), formation of receptor complexes (66) and with providing a functional molecular configuration for membrane proteins (118). The ganglioside-protein association would be mediated
by salt bridges and/or hydrogen bonds rather than by covalent bond formation. Examples of a direct association recently have been shown for: the Paul-Bunnel antigen in bovine erythrocytes (203) the P2 myelin-specific protein of peripheral nerves (118) and human lymphocyte mIg (35). This latter report and the results from the present study are based on cocapping data, whereas biochemical techniques were used in the former two studies.

A series of monoclonal antibodies to human B₂-microglobulin and to "Ia-antigen" were used to study the possible association of these HLA related molecules with the G₅₁-like antigenic determinant. B₂-microglobulin is not inserted into the plasma membrane (176), and thus it would not directly facilitate an association with cytoskeletal elements; whereas, this molecule's association with the membrane inserted HLA associated antigens (32) would provide for an indirect means of membrane ganglioside communication with the cytoplasm. A second reason for choosing B₂-microglobulin is that its molecular weight of 11,700 daltons approximates the first immunoprecipitation peak seen in Figure 5. There is no apparent cocapping of B₂-microglobulin with the G₅₁-like antigenic determinant, or visa versa. Pretreatment of cells with VCN did not alter this finding. Eventhough there is no direct association between these two molecules it does not eliminate a possible association between the heavy and/or light chains of the HLA molecules, those regions of mIg not homologous with B₂-microglobulin, or the G₅₁-like antigenic determinant.
The redistribution of mIg into a cap, following the binding of anti-IgG to the cell surface, results in a signal being transmitted from the membrane to the cytoplasm (160). Curtain (35) considered that this signal is cAMP and that it is derived from an activation of adenyl cyclase by a change in the local membrane lipid environment. This change refers to the formation of lipid domain consisting of the GSLs linked to the mIg by hydrogen bonds. Curtain (35) found that both G\textsubscript{M1} and cerebroside were able to cocap with the mIg, and that a similar concentration of membrane cAMP was directly associated with anti-G\textsubscript{M1} induced caps. The results in Table 11 confirm this association between mIg and the G\textsubscript{M1}-like antigenic determinant. The ability of VCN to completely disrupt this association indicates that sialic acid residues mediate this association. Thus a signal is generated by a local change in membrane fluidity, brought about by an ordering of the lipids between the two layers, which in turn alters the molecular configuration of a membrane protein in such a way as to activate it. This conclusion refers only to a molecule or molecules carrying the G\textsubscript{M1}-like antigenic determinants to which the antibodies to G\textsubscript{M1} bind. This reservation is based on the results of Craig and Cuatrecasas (31) and Sedlacek et al. (168) who were unable to show a cocapping of G\textsubscript{M1} and mIg when capping is induced with cholera toxin. The results on Table 11 and those of Curtain (35) are based on cocapping induced with anti-Ig and not anti-G\textsubscript{M1} which may indicate that an association only results from a clustering of the mIg prior to cap formation.
HLA-DR, or "Ia-like", glycoprotein molecules are found in the membrane of both human T and B lymphocytes (3, 84, 159, 176). Parish and coworkers (72, 73, 130, 131, 132) have described a carbohydrate defined Ia antigen in both mice and man. This latter antigen is considered to have the properties of a macroganglioside (72). Parish and McKenzie (132) have proposed that the association of the protein and macroganglioside defined Ia antigens is one of an enzyme and its substrate. Such a relationship would explain the results seen in this study (Table 13). In this manner the G\(\text{M}_1\)-like antigenic determinant would be part of the substrate molecule for an enzyme carrying the Ia antigen. The ability of the monoclonal antibody to reduce the binding of antibodies to G\(\text{M}_1\), but not visa versa, indicates that the antigenic epitope on the Ia-protein is in close approximation to the site of association. Binding of the monoclonal antibody may alter the molecular configuration of the protein-G\(\text{M}_1\) association site thus reducing the exposure of the G\(\text{M}_1\)-like antigenic determinant for subsequent binding of anti-G\(\text{M}_1\). The reverse is not the case; antibodies to G\(\text{M}_1\) bind to multiple epitopes on the carbohydrate which tends to anchor it and prevent its distortion following monoclonal antibody binding to the Ia-protein. This explanation also suggests that the association between the Ia protein and the G\(\text{M}_1\)-like determinant molecule(s) may not be on a one-to-one basis, and that there are more substrate molecules than enzyme molecules. In a significant number of cases, VCN pretreatment of cells disrupts the cocapping of both determinants indicating that the association involves sialic acid residues. The cryptic G\(\text{M}_1\)-like
antigenic determinants exposed by VCN probably lies deeper in the glycocalyx and not necessarily in association with the Ia-protein. Another, and a more simplified, explanation of these results would be for the $G_M^1$-like antigenic determinant to lie on one of the two glycoprotein chains binding the monoclonal antibody. Even though this latter explanation can not be totally discounted, it does not account for a lack of a protein-associated peak following immunoprecipitation of labelled membrane lysates with antibodies to $G_M^1$ (Figure 5). Functionally, an association of either form would facilitate the transduction of a signal from the membrane to the cytoplasm. Ligand binding to the ganglioside would induce a conformational change in the protein Ia molecule necessary for the signal to transmitted. Binding could also permit cap formation and a resultant signal transduction due to an association of the Ia protein with cytoskeletal elements.

The cap of the $G_M^1$-like antigenic determinant, as well as membrane incorporated exogenous $G_M^1$, is lost from the cell by both endocytosis and shedding. Ackerman et al. (1) have shown that endocytosis of the cholera toxin-$G_M^1$ complex is accomplished by membrane invagination. The fact that endocytosis does not require cap formation (160) was observed in this study. Shedding of the capped complex was often seen (Plate IV), to be accompanied by the formation of a uropod over which the cap lies. A similar observation has been made with anti-$G_M^1$ induction of caps on rat thymocytes (169) and anti-Ig induced capping of mIg from various species (160). With time, it is apparent that the organelle rich uropod is constricted to the point of being totally discharged from
the cell surface. No cell surface staining remains following the release of the vesicle. As noted with the formation of mIg containing uropods (160), capping of the G\textsubscript{M1}-like antigenic determinant precedes the formation of the uropod. This change in cell morphology results in the "hand mirror" appearance characteristic of cell motility. Even though I did not study anti-G\textsubscript{M1} induction of lymphocyte motility, I observed that the presence of colchicine resulted in four times as many uropod containing cells as compared to untreated cells. Schreiner and Unanue (160) reported that colchicine pretreatment of B cells resulted in an increase in anti-Ig stimulated motility. Morphological changes were primarily seen when capping was induced with a second antibody. It is most likely that the second antiserum amplifies the membrane perturbations leading to the formation of uropods and their subsequent pinching off.
**TABLE 1**

**STRUCTURES OF GANGLIOSIDES**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosyl Ceramide</td>
<td>Gal(Bl-1)-Ceramide</td>
</tr>
<tr>
<td>Gm4</td>
<td>NANA((\alpha)2-3)Gal(Bl-1)-Ceramide</td>
</tr>
<tr>
<td>Gm3 (Hematoside)</td>
<td>NANA((\alpha)2-3)Gal(Bl-4)Glc(Bl-1)-Ceramide</td>
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<tr>
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</tr>
<tr>
<td>Gm1</td>
<td>Gal(Bl-3)GalNAc(Bl-4)Gal(Bl-4)Glc(Bl-1)-Ceramide</td>
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<td>(\beta)2NANA</td>
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<tr>
<td></td>
<td>(\beta)2NANA</td>
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<tr>
<td>D1a</td>
<td>Gal(Bl-3)GalNAc(Bl-4)Gal(Bl-4)Glc(Bl-1)-Ceramide</td>
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<tr>
<td></td>
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<tr>
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</tr>
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<td></td>
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<tr>
<td>T1a</td>
<td>Gal(Bl-3)GalNAc(Bl-4)Gal(Bl-4)Glc(Bl-1)-Ceramide</td>
</tr>
<tr>
<td></td>
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<td>(\beta)2NANA(8-2(\alpha))NANA</td>
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Gal - Galactose
Glu - Glucose
GalNAc - N-Acetyl-Galactosamine
NANA - Sialic Acid (N-Acetyl Neuramic Acid)
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<tr>
<th>ANTISERUM</th>
<th>G_M1</th>
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<th>G_D1a</th>
<th>G_H1b</th>
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<tr>
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</tr>
<tr>
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<td>15P-UB^f</td>
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<tr>
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<td>17P1UB</td>
<td>50.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a ug Protein at 50% lysis
b P - Pool
c AB - Serum was affinity purified on a BSA-Sepharose 4B Column
d g - Heat inactivated serum sample pool
e AP - Serum was affinity purified on a G_M1-Silica Gel Column
f UB - Unbound fraction of serum sample passed over a G_M1-Silica gel column
8 ND - Not Done
### TABLE 3

**DEGREE OF CROSSREACTIVITY OF 14P SERUM**

**AS A FUNCTION OF ANTIGEN CONCENTRATION**

<table>
<thead>
<tr>
<th>ANTIGEN CONCENTRATION (ng/tube)</th>
<th>COMPLEMENT FIXATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>GANGLIOSIDE</strong></td>
</tr>
<tr>
<td></td>
<td>Gm1  Gm2  Gm3  Gm4  Gm1a  Gm1b  Gt1</td>
</tr>
<tr>
<td>.0146</td>
<td>93.0  91.0  25.0  43.0  32.0  96.0  21.0</td>
</tr>
<tr>
<td>.0730</td>
<td>100.0 99.0  61.0  81.0  69.0  95.0  53.0</td>
</tr>
<tr>
<td>.1460</td>
<td>100.0 99.0  92.0  94.0  89.0  92.0  74.0</td>
</tr>
<tr>
<td>.3650</td>
<td>100.0 95.0  98.0  95.0  88.0  90.0  75.0</td>
</tr>
</tbody>
</table>

*a 1:500 Dilution of 14P Serum (9.3 ug protein/ml)*

### TABLE 4

**DEGREE OF CROSSREACTIVITY OF 14P IgG**

**AS A FUNCTION OF ANTIGEN CONCENTRATION**

<table>
<thead>
<tr>
<th>ANTIGEN CONCENTRATION (ng/tube)</th>
<th>COMPLEMENT FIXATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>GANGLIOSIDE</strong></td>
</tr>
<tr>
<td></td>
<td>Gm1  Gm2  Gm3  Gm4  Gm1a  Gm1b  Gt1</td>
</tr>
<tr>
<td>.0146</td>
<td>87.0  ND  41.0  42.0  2.0  53.0  ND</td>
</tr>
<tr>
<td>.0730</td>
<td>96.0  ND  60.0  55.0  26.0  60.0  ND</td>
</tr>
<tr>
<td>.1460</td>
<td>97.0  ND  75.0  68.0  38.0  71.0  ND</td>
</tr>
</tbody>
</table>

*a 1:250 dilution of 14P IgG (1.6 ug protein/ml)*

b ND - Not Done
TABLE 5

PERCENTAGE OF LYMPHOCYTE POPULATIONS EXPRESSING THE GM1-LIKE ANTIGENIC DETERMINANT

<table>
<thead>
<tr>
<th>CELL SOURCE</th>
<th>SERUM SAMPLE</th>
<th>CONC. RANGE (µg)</th>
<th>PERCENT STAINING</th>
</tr>
</thead>
<tbody>
<tr>
<td>THYMUS</td>
<td>14P Ig-UC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(20-10)</td>
<td>20.0±5.0</td>
</tr>
<tr>
<td></td>
<td>NRS Ig-UC</td>
<td>(20-10)</td>
<td>5.0±4.0</td>
</tr>
<tr>
<td>FETAL CORD BLOOD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15P-AP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(50-20)</td>
<td>29.0±12.8</td>
</tr>
<tr>
<td></td>
<td>17P&lt;sub&gt;1&lt;/sub&gt;-AP</td>
<td>(50-20)</td>
<td>41.9±27.3</td>
</tr>
<tr>
<td>ADULT PERIPHERAL BLOOD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>LYMPHOCYTES</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14P Ig-UC</td>
<td>(20-10)</td>
<td>20.3±9.3</td>
</tr>
<tr>
<td></td>
<td>14P&lt;sub&gt;B&lt;/sub&gt; Ig-UC</td>
<td>(20)</td>
<td>7.7±2.7</td>
</tr>
<tr>
<td></td>
<td>15P-AP</td>
<td>(100-50)</td>
<td>11.0±2.6</td>
</tr>
<tr>
<td></td>
<td>16P&lt;sub&gt;4&lt;/sub&gt;-AP</td>
<td>(100-10)</td>
<td>11.6±2.6</td>
</tr>
<tr>
<td></td>
<td>17P&lt;sub&gt;1&lt;/sub&gt;-AP</td>
<td>(50)</td>
<td>7.7±1.9</td>
</tr>
<tr>
<td></td>
<td>N.W. ADHERENT CELLS</td>
<td>15P-AP</td>
<td>(100-50)</td>
</tr>
<tr>
<td></td>
<td>16P&lt;sub&gt;4&lt;/sub&gt;-AP</td>
<td>(50)</td>
<td>39.8±2.5</td>
</tr>
<tr>
<td></td>
<td>N.W. NONADHERENT CELLS</td>
<td>16P&lt;sub&gt;4&lt;/sub&gt;-AP</td>
<td>(100-50)</td>
</tr>
<tr>
<td></td>
<td>PLASTIC NONADHERENT CELLS</td>
<td>15P-AP</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>E ROSETTE&lt;sup&gt;+&lt;/sup&gt; CELLS</td>
<td>15P-AP</td>
<td>(100-50)</td>
</tr>
<tr>
<td></td>
<td>sIg&lt;sup&gt;+&lt;/sup&gt; CELLS&lt;sup&gt;f&lt;/sup&gt;</td>
<td>15P-AP</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16P&lt;sub&gt;4&lt;/sub&gt;-AP</td>
<td>(100-50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17P&lt;sub&gt;1&lt;/sub&gt;-AP</td>
<td>(100)</td>
</tr>
</tbody>
</table>

<sup>a</sup>UC - Serum Ig sample was ultracentrifuged
<sup>b</sup>- Mononuclear cell preparation from fetal cord and adult peripheral blood samples.
<sup>c</sup> AP - Serum was affinity purified on a GM1-silica gel column
<sup>d</sup> PIB - Preimmune bleed
<sup>e</sup> N.W. - Nylon wool
<sup>f</sup> sIg<sup>+</sup> - Cells expressing immunoglobulin on their surface
TABLE 6

EFFECT OF ENZYME ON CELLULAR EXPRESSION OF THE G\textsubscript{M1}-LIKE ANTIGENIC DETERMINANT

<table>
<thead>
<tr>
<th>SERUM</th>
<th>EXP\textsuperscript{a}</th>
<th>SBSS</th>
<th>VCN</th>
<th>PROTEASE</th>
<th>TRYPsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ug)</td>
<td>#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13F Ig-UC\textsuperscript{b}</td>
<td>1</td>
<td>10.5</td>
<td>ND</td>
<td>9.9</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.0</td>
<td>52.4</td>
<td>2.4</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.0</td>
<td>88.7</td>
<td>14.6</td>
<td>15.2</td>
</tr>
<tr>
<td>NRS Ig-UC (30 ug)</td>
<td>1</td>
<td>12.0</td>
<td>ND</td>
<td>3.9</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.0</td>
<td>4.0</td>
<td>4.0</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.3</td>
<td>51.0</td>
<td>3.0</td>
<td>7.0</td>
</tr>
<tr>
<td>THYMOCYTES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14P Ig-AB\textsuperscript{d} (20 ug)</td>
<td>1</td>
<td>4.1</td>
<td>69.4</td>
<td>14.1</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.8</td>
<td>ND</td>
<td>11.6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19.2</td>
<td>ND</td>
<td>12.6</td>
<td>ND</td>
</tr>
<tr>
<td>NRS Ig-AB (20 ug)</td>
<td>1</td>
<td>2.0</td>
<td>15.7</td>
<td>0.5</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.6</td>
<td>35.8</td>
<td>ND</td>
<td>6.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Each experiment represents a different individual
\textsuperscript{b} UC - Serum Ig sample was ultracentrifuged
\textsuperscript{c} ND - Not Determined
\textsuperscript{d} AB - Serum Ig sample was affinity purified on a BSA-Sepharose 4B Column.
### TABLE 7

**EFFECT OF VCN ON THE EXPRESSION OF THE G_{M1}-LIKE ANTIGENIC DETERMINANT ON LYMPHOCYTES FROM DIFFERENT SOURCES**

<table>
<thead>
<tr>
<th>PERCENT STAINING</th>
<th>PBMC_{a}</th>
<th>FCBMC_{b}</th>
<th>SPLEEN MC_{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Stain</td>
<td>Range</td>
<td>% Stain</td>
</tr>
<tr>
<td>14P Ig-ABd</td>
<td>87.8±14.8</td>
<td>61.5-96.5</td>
<td>99.0±1.0</td>
</tr>
<tr>
<td>(5ug-10ug)</td>
<td></td>
<td></td>
<td>81.9±13.8</td>
</tr>
<tr>
<td>(5ug-20ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14PIB_{e} Ig-AB</td>
<td>23.4±17.8</td>
<td>7.0-47.0</td>
<td>60.7±10.2</td>
</tr>
<tr>
<td>(5ug-10ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5ug-20ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRS Ig-AB</td>
<td>35.7±15.9</td>
<td>19.8-51.5</td>
<td></td>
</tr>
<tr>
<td>(5ug-20ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15P-AP_{f}</td>
<td>36.4±32.2</td>
<td>16.0-89.3</td>
<td>38.9±5.4</td>
</tr>
<tr>
<td>(5ug-20ug)</td>
<td></td>
<td></td>
<td>52.6±33.6</td>
</tr>
<tr>
<td>(5ug-100ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16P_{4}-AP</td>
<td>96.6±1.9</td>
<td>94.5-98.0</td>
<td></td>
</tr>
<tr>
<td>(50ug-100ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16P_{4}-IgG</td>
<td>22.2±4.8</td>
<td>18.0-28.0</td>
<td>65.3±0.2</td>
</tr>
<tr>
<td>(20ug-50ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(100ug-200ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17P_{1}-AP</td>
<td>19.6±5.7</td>
<td>13.2-26.8</td>
<td></td>
</tr>
<tr>
<td>(10ug-50ug)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*a* PBMCs - Peripheral blood mononuclear cells  
*b* FCBMCs - Fetal cord blood mononuclear cells  
*c* Spleen MCs - Spleenic mononuclear cells  
*d* AB - Serum was affinity purified on a BSA-Sepharose 4B Column  
*e* PIB - Preimmune serum  
*f* AP - Serum was affinity purified on a G_{M1}-Silica Gel Column
<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Serum (µg)</th>
<th>SBSS</th>
<th>VCN</th>
<th>GM1</th>
<th>GT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14P Ig-UC&lt;sup&gt;b&lt;/sup&gt; (115)</td>
<td>31.0</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
<td>28.5</td>
</tr>
<tr>
<td></td>
<td>NRS Ig-UC (74)</td>
<td>1.8</td>
<td>ND</td>
<td>8.8</td>
<td>8.0</td>
</tr>
<tr>
<td>2</td>
<td>14P Ig-AB&lt;sup&gt;d&lt;/sup&gt; (10)</td>
<td>13.5</td>
<td>61.5</td>
<td>77.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(50)</td>
<td>51.0</td>
<td>100</td>
<td>98.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>57.7</td>
<td>100</td>
<td>88.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>NRS Ig-AB (10)</td>
<td>3.9</td>
<td>28.5</td>
<td>6.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(50)</td>
<td>12.0</td>
<td>68.3</td>
<td>15.8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>13.0</td>
<td>99.5</td>
<td>13.2</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>15P-AP&lt;sup&gt;e&lt;/sup&gt; (50)</td>
<td>9.4</td>
<td>26.6</td>
<td>15.8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>11.3</td>
<td>29.6</td>
<td>13.6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>17P&lt;sub&gt;1&lt;/sub&gt;-AP (50)</td>
<td>8.5</td>
<td>20.4</td>
<td>25.6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>8.0</td>
<td>26.8</td>
<td>59.6</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>16P4-AP&lt;sup&gt;f&lt;/sup&gt; (100)</td>
<td>15.6±4.5</td>
<td>96.8±3.3</td>
<td>81.3±1.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lymphocytes were derived from PBMCs
<sup>b</sup> UC - Serum Ig sample was ultracentrifuged
<sup>c</sup> ND - Not Determined
<sup>d</sup> AB - Serum was affinity purified on a BSA - Sepharose 4B Column
<sup>e</sup> AP - Serum was affinity purified on a G<sub>M1</sub>-Silica Gel Column
<sup>f</sup> The values for experiments 1 through 3 represent a single observation. The results in experiment 4 represent the mean and S.D. percent staining for four experiments using the same antiserum.
# TABLE 9

RATE OF CAP FORMATION OF THE G\textsubscript{M1}-LIKE ANTIGENIC DETERMINANT

<table>
<thead>
<tr>
<th>Time(^a) (min)</th>
<th>PERCENT CAPPING(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CELL PRETREATMENT</td>
</tr>
<tr>
<td></td>
<td>SBSS</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10.3</td>
</tr>
<tr>
<td>10</td>
<td>12.7</td>
</tr>
<tr>
<td>20</td>
<td>29.1</td>
</tr>
</tbody>
</table>

\(^a\) PBMCs were reacted with 50 ug 16F\(_4\) for 60 minutes at 0-4°C, and stained with FITC-F(ab\(^+\))\(_2\)-goat anti-rabbit IgG F(ab\(^+\))\(_2\) for 60 minutes at 0-4°C. Capping was induced by incubating the cells at 37°C and was stopping by fixation with 4% formaldehyde in SBSS.

\(^b\) % of staining cells exhibiting cap formation.
<table>
<thead>
<tr>
<th>PHARMACOLOGICAL REAGENT (Concentration)</th>
<th>PERCENT CAPPING INHIBITION²</th>
<th>CELL PRETREATMENT</th>
<th>SBSS</th>
<th>VCN</th>
<th>GM₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaN₃ (0.01M)</td>
<td></td>
<td>83.0</td>
<td>92.0</td>
<td>90.0</td>
<td></td>
</tr>
<tr>
<td>NaF (0.1M)</td>
<td></td>
<td>100</td>
<td>93.0</td>
<td>73.0</td>
<td></td>
</tr>
<tr>
<td>2,4-DNP (5X10⁻⁴M)</td>
<td></td>
<td>100</td>
<td>89.0</td>
<td>77.0</td>
<td></td>
</tr>
<tr>
<td>2-dexoxy-D-glucose (0.05M)</td>
<td></td>
<td>90.0</td>
<td>92.0</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide (10ug/ml)</td>
<td></td>
<td>-45.0d</td>
<td>6.0</td>
<td>63.0</td>
<td></td>
</tr>
<tr>
<td>Colchicine (10⁻⁵M)</td>
<td></td>
<td>-5.0</td>
<td>-20.0</td>
<td>-92.0</td>
<td></td>
</tr>
<tr>
<td>Cytochalasin B (10ug/ml)</td>
<td></td>
<td>100</td>
<td>-22.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1:1 Col./Cyto. B</td>
<td></td>
<td>100</td>
<td>65.0</td>
<td>57.0</td>
<td></td>
</tr>
<tr>
<td>Lidocaine (10⁻³ M)</td>
<td></td>
<td>41.0</td>
<td>36.0</td>
<td>58.0</td>
<td></td>
</tr>
</tbody>
</table>

² PBMCs were reacted with 50 ug of 16P₄-AP and stained with FITC-F(ab')₂ goat anti-rabbit IgG F(ab')₂. Capping was stopped by fixation with 4% formaldehyde in SBSS.

b % Inhibition = \( \frac{\% \text{NaN₃ CAPPED}}{\% \text{SBSS CAPPED}} \times 100 \)

c % INHIBITION = \( \frac{\% \text{Experimental Noncapped} - \% \text{SBSS Noncapped}}{\% \text{NaN₃ Noncapped} - \% \text{SBSS Noncapped}} \times 100 \)

² A negative reading indicates an enhancement of cap formation.
### TABLE 11

**G	extsubscript{M1}-LIKE ANTIGENIC DETERMINANT COCAPPING WITH MEMBRANE Ig**

<table>
<thead>
<tr>
<th>CELL SOURCE</th>
<th>SERUM</th>
<th>TOTAL Ig+ %</th>
<th>TOTAL Ia+ %</th>
<th>% G	extsubscript{M1}+ CELLS COCAPPED</th>
<th>% Ig+ CAP</th>
<th>% CSb COCAPPED</th>
<th>% G	extsubscript{M1}+ CAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMCS SBSS</td>
<td>16P4-AP (100)</td>
<td>14.0</td>
<td>4.0</td>
<td>17.9</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>VCN</td>
<td>93.5</td>
<td>10.0</td>
<td>10.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NWAC</td>
<td>SBSS 16P4-AP (100)</td>
<td>37.5</td>
<td>47.5</td>
<td>45.3</td>
<td>41.2</td>
<td>0</td>
<td>7.4</td>
</tr>
</tbody>
</table>

\(\text{a} \text{- Cell Pretreatment} \)
\(\text{b} \text{- Costained} \)
\(\text{c} \text{- NWAC Nylon Wool Adherent Cells} \)

### TABLE 12

**G	extsubscript{M1}-LIKE ANTIGENIC DETERMINANT COCAPPING WITH B\textsubscript{2}-MICROGLOBULIN**

<table>
<thead>
<tr>
<th>CELL SOURCE</th>
<th>SERUM</th>
<th>TOTAL G	extsubscript{M1}+ %</th>
<th>TOTAL B\textsubscript{2}-M+ %</th>
<th>% G	extsubscript{M1}+ CELLS COCAPPED</th>
<th>% B\textsubscript{2}-M+ CAP</th>
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<tr>
<td>PBMCS SBSS</td>
<td>16P4-AP (100)</td>
<td>22.0</td>
<td>94.0</td>
<td>95.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>VCN</td>
<td>100</td>
<td>98.0</td>
<td>98.0</td>
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</tr>
<tr>
<td>PBMCS SBSS</td>
<td>16P4-AP (100)</td>
<td>15.5</td>
<td>6.5</td>
<td>22.6</td>
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\(\text{a} \text{- Cell Pretreatment} \)
\(\text{b} \text{- Costained} \)
\(\text{c} \text{- B\textsubscript{2}-M B\textsubscript{2}-Microglobulin} \)
<table>
<thead>
<tr>
<th>CELL SOURCE</th>
<th>SERUM</th>
<th>SBSS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TOTAL</th>
<th>TOTAL</th>
<th>G&lt;sub&gt;M1&lt;/sub&gt;</th>
<th>Ia</th>
<th>G&lt;sub&gt;M1&lt;/sub&gt;</th>
<th>Ia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VCN</td>
<td></td>
<td>G&lt;sub&gt;M1&lt;/sub&gt;</td>
<td>Ia+</td>
<td>Ia+</td>
<td>CS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>COCAPPED</td>
<td>CAP</td>
</tr>
<tr>
<td>NWA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>SBSS 16F&lt;sub&gt;4&lt;/sub&gt;-AP (50)</td>
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<td>32.0</td>
<td>71.4</td>
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<td></td>
<td>VCN</td>
<td>93.0</td>
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<td>NWA</td>
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<td></td>
<td>VCN</td>
<td>96.0</td>
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<td>43.2</td>
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<sup>a</sup> Cell Pretreatment
<sup>b</sup> CS Costained
<sup>c</sup> NWA Nylon Wool Adherent Cells
**TABLE 14**

**COMPARISON OF mIgG and Gm1 CAPPING**

<table>
<thead>
<tr>
<th>CAPPING</th>
<th>mIg&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gm1 CAPPING</th>
<th>VCN</th>
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<tr>
<td></td>
<td>CAPPING</td>
<td>INTRINSIC</td>
<td>EXPOSED</td>
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<tr>
<td>Ligand Multivalency</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Ligand Monovalency</td>
<td>No</td>
<td>No</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Energy Requirement</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cytochalasin B Inhibition</td>
<td>Yes/No</td>
<td>Yes</td>
<td>Yes/No</td>
</tr>
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<td>Colchicine Enhancement</td>
<td>Yes/No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lidocaine Inhibition</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Spontaneous Reversal</td>
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<td>No</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Schreiner and Unanue (160)

<sup>b</sup> Not Determined
Figure 1
Structure of $C_{H1}$

* Brunngraber (23) and Fishman & Brady (52)
FIGURE 2: Effect of Enzyme Treatment on the Expression of the Cryptic and Noncryptic G\textsubscript{M1}-like Antigenic Determinants. Adult peripheral blood mononuclear cells were pretreated with SBSS (noncryptic G\textsubscript{M1}-like antigenic determinant) or neuraminidase (VCN - cryptic G\textsubscript{M1}-like antigenic determinant) followed by either 1.0 mg or 2.5 mg of trypsin or protease. Immunofluorescent staining was performed with either the immunoglobulin (Ig) fraction of an anti-G\textsubscript{M1} serum pool (14P-Ig AB) or with the preimmune Ig fraction (14PIB-Ig AB) of the same antiserum. Only positively stained lymphocytes were counted.
<table>
<thead>
<tr>
<th>SERUM SAMPLE</th>
<th>CELL PRETREATMENT</th>
<th>ENZYME TREATMENT</th>
<th>PERCENT STAINING</th>
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<tr>
<td>14PIg-AB</td>
<td>SBSS</td>
<td>TRYPsin (1.0)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PROTEASE (1.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCN</td>
<td>TRYPsin (1.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PROTEASE (1.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SBSS</td>
<td>TRYPsin (2.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PROTEASE (2.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCN</td>
<td>TRYPsin (2.5)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>PROTEASE (2.5)</td>
<td></td>
</tr>
<tr>
<td>14PIB Ig-AB</td>
<td>SBSS</td>
<td>TRYPsin (1.0)</td>
<td></td>
</tr>
<tr>
<td>(10 ug)</td>
<td></td>
<td>PROTEASE (1.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCN</td>
<td>TRYPsin (1.0)</td>
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<tr>
<td></td>
<td></td>
<td>PROTEASE (1.0)</td>
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<tr>
<td></td>
<td>SBSS</td>
<td>TRYPsin (2.5)</td>
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<tr>
<td></td>
<td></td>
<td>PROTEASE (2.5)</td>
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**FIGURE 2:** EFFECT OF ENZYME TREATMENT ON THE EXPRESSION OF THE CRYPTIC AND NONCRYPTIC GM1-LIKE ANTIGENIC DETERMINANT
FIGURE 3: Trypsin Sensitivity of the VCN Exposed Cryptic GM1-like Antigenic Determinant. Adult peripheral blood mononuclear cells were treated with neuraminidase (VCN) and then with trypsin (1.0 mg) or in the reverse order. Immunofluorescent staining used either 10 ug or 50 ug per tube of an anti-GM1 serum immunoglobulin fraction (14P-Ig AB) or the Ig fraction from a normal rabbit serum (NRS-Ig AB). Only positively stained lymphocytes were counted.
## FIGURE 3: TRYPSIN SENSITIVITY OF THE VCN EXPOSED CYRPTIC G\(_{M1}\)-LIKE ANTIGENIC DETERMINANT

<table>
<thead>
<tr>
<th>SERUM SAMPLE</th>
<th>ENZYME TREATMENT</th>
<th>PERCENT STAINING</th>
</tr>
</thead>
<tbody>
<tr>
<td>14PIg-AB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ug</td>
<td>VCN-TRYPSIN</td>
<td>10 30 50 70 90</td>
</tr>
<tr>
<td></td>
<td>TRYPsin-VCN</td>
<td></td>
</tr>
<tr>
<td>50 ug</td>
<td>VCN-TRYPSIN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRYPsin-VCN</td>
<td></td>
</tr>
<tr>
<td>NRS Ig-AB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ug</td>
<td>VCN-TRYPSIN</td>
<td>10 30 50</td>
</tr>
<tr>
<td></td>
<td>TRYPsin-VCN</td>
<td></td>
</tr>
<tr>
<td>50 ug</td>
<td>VCN-TRYPSIN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRYPsin-VCN</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 4: The Effect of Removing Sugar Units from the Cell Surface on
the Expression of the Cryptic and Noncryptic G_{M1}-like Antigenic
Determinants. Adult peripheral blood mononuclear cells were pretreated
with either SBSS (noncryptic G_{M1}-like antigenic determinant) or
neuraminidase (VCN - cryptic G_{M1}-like antigenic determinant) followed by
one of three glycolytic reagents. Immunofluorescent staining was
performed with 100 ug/tube of an affinity purified anti-G_{M1} serum pool
(17P1). Only positively staining lymphocytes were counted.
Figure 4: The effect of removing sugar units from the cell surface on the expression of the cryptic and noncryptic GM1-like antigenic determinants.
FIGURE 5: Immunoprecipitation of $^3$H-Labeled PBMCs. Adult peripheral blood mononuclear cells (PBMCs) were pretreated with neuraminidase and galactose oxidase and then labeled with $^3$H sodium borohydride. Membrane extracts were pretreated with the IgG fraction of NRS (○—○) or 16P$_4$ (□—□) and immunoprecipitated with SPA. Control PBMCs were labeled with $^3$H-G$_{M1}$ (●—●) and the treated with 16P$_4$ IgG and SPA.

The molecular weight standards: a (phosphorylase B - 94,000 daltons), b (BSA - 68,000 daltons), c (ovalbumin - 43,000 daltons), d (carbonic anhydrase - 30,000 daltons), e (soybean trypsin inhibitor - 21,000 daltons), and f (lysozyme - 14,300 daltons).
FIGURE 5: IMMUNOPRECIPITATION OF $^3$H-Labeled PBMCs
FIGURE 6: Immunoprecipitation of 125I Labeled PBMCs. Membrane extracts of peripheral blood mononuclear cells (PBMCs), labeled with 125I, were pretreated with PBS (---), or the IgG fraction of NRS (O--O), or 16P4 (X--X). Molecular weight standards: a (phosphorylase B - 94,000 daltons), b (BSA - 68,000 daltons), c (ovalbumin - 43,000 daltons), d (carbonic anhydrase - 30,000 daltons), e (soybean trypsin inhibitor - 21,000 daltons), and f (lysozyme - 14,300 daltons).
FIGURE 6: IMMUNOPRECIPITATION OF 125I-Labeled PBMCs
PLATE I: Immunoelectrophoresis of Anti-GM1 Serum. A. Anti-GM1 16P4IgG. Wells 1 and 3 contain 16P4IgG. B. Anti-GM1 16P4 affinity purified (AP). Wells 1 and 3 contain 16P4-AP. Wells 2 and 4 in A and B contain normal rabbit serum. The upper trough contains goat anti-rabbit IgG-Fc, while the lower trough contains goat anti-rabbit whole serum. White arrow represents albumin.
PLATE I: Immunoelectrophoresis of Anti-\(\text{GM}_1\) Serum
PLATE II: Immunofluorescent Staining of Human Lymphocytes with Anti-\(G_{M1}\) Serum. 1) Continuous run of staining-cells were pretreated with neuraminidase; 2) Granular staining pattern-cells were untreated; 3) Large patches of stain-cells were pretreated with \(G_{M1}\).
PLATE II: Immunofluorescent Staining of Human Lymphocytes with Anti-CH1 Serum
PLATE III: Capping of the G\textsubscript{M1}-like Antigenic Determinant on the Surface of Human Lymphocytes. Arrowhead (1) indicates the site of G\textsubscript{M1}-like antigenic determinant capping (2) at the pole facing the nuclear hof region of the lymphocyte cells were untreated. Capping proceeds from the initial binding of antibodies (3), to the formation of aggregates (4 and 5) and finally to the formation of a single cap (6). This process occurs despite the fact that the cells are untreated (5) or pretreated with neuraminidase (3 and 6) or G\textsubscript{M1} (4).
PLATE III: Capping of the GM1-like Antigenic Determinant on the Surface of Human Lymphocytes
PLATE IV: Formation of a Uropod Containing the Gm-like Antigenic Determinant on the Surface of Human Lymphocytes. Capping of the Gm-like antigenic determinant often results in the formation of a uropod (arrowhead 1, 3, 5, and 7). Immunofluorescent staining is concentrated at the site of the Uropod (2, 4, 6, and 8—correspond to the respective cell in the left-hand column). The uropod often contains cytoplasmic organelles (5 and 7). The initial site of uropod formation is a cap (1) and progresses from a small surface blebs (3) to various sizes of uropods, which can give the appearance of pinching off (7). This process is increased in the presence of colchicine (7 and 8).
PLATE IV: Formation of Uropods Containing the G_{M1}-like Antigenic Determinant on the Surface of Human Lymphocyte.
PART 2
INTRODUCTION

Antibodies to membrane glycoconjugates have been used to distinguish subpopulations of human T and B lymphocytes (21,38,144,164,165). Functions have yet to be ascribed to the molecules, principally glycoproteins (21,38,116), on which these antigenic determinants are located. Part 1 of this dissertation demonstrated that antibodies to the $G_m^{1}$ ganglioside were capable of differentiating subpopulations of human T and B lymphocytes. The molecule, or molecules, carrying this $G_m^{1}$-like antigenic determinant was found to have the properties and membrane behavior of a ganglioside. As a group, gangliosides have been found in the plasma membrane of lymphocytes from both mammalian and nonmammalian species (103,117,122,132,151); however, so far their antigenic expression has only been demonstrated for rat (7), murine (67,184), and human (165) lymphocytes. To date, the only functionally distinct subpopulation of lymphocytes defined by antibodies to a glycosphingolipid is the natural killer (NK) cell in both mice (83,210) and man (Herberman - personal communication). These antibodies recognize the neutral glycosphingolipid, asialo-$G_m^{1}$. As noted in Part 1, antibodies to $G_m^{1}$ cross react with asialo-$G_m^{1}$ (119), which is considered here to be a molecule carrying the $G_m^{1}$-like antigenic determinant. The function, or
functions, of asialo-GL$_1$ in the plasma membrane of the NK cell is unknown, but it or a similar GL$_1$-like antigenic determinant may serve as part of the receptor complex mediating the binding of interferons to the NK cell surface.

In man, NK cells are a morphologically distinct group of large granular lymphocytes (189) which are ultrastructurally similar to monocytes (135) and carry antigenic determinants of both T cells and monocytes (Herberman - personal communication). Without prior antigenic stimulation, these cells demonstrate spontaneous cytolytic activity toward a wide range of malignant cells (189). The cytolytic activity of NK cells is augmented, both in vitro and in vivo, by the addition of interferons (41,43,47,65,215) or inducers of interferon release (43,44,65,111,141). All three forms of interferon: fibroblast (215), leukocyte (47) and immune (41) are capable of augmenting human NK cell activity.

In order for interferon to exert an effect, it must first bind to a high affinity receptor in the NK cell's plasma membrane (9). Early studies showed that a preincubation of either murine (13,14) or human (94,198,199) interferon with gangliosides resulted in a neutralization of interferon's ability to induce an antiviral state. Recently, Whisler (unpublished results) has neutralized the ability of interferon to augment NK cell activity by preincubating the cells with gangliosides. Whereas only murine fibroblast interferon binds to gangliosides (6), both human fibroblast and leukocyte interferons have been shown to bind various gangliosides (94,198). Vengris et al. (198) were able to
correlate the sensitivity of cells to interferon with the ganglioside pattern of the membrane. These investigators were able to restore interferon sensitivity by reincubating the cells with gangliosides. Friedman and coworkers (56, 57, 58, 66) have shown that the membrane receptor for interferon is a complex consisting of a "high affinity" glycoprotein in addition to a "low affinity" ganglioside responsible for signal transduction.

Murine fibroblast interferon primarily binds to $G_m^2$ and $G_t^1$ (13); whereas, human interferons bind to several gangliosides (198) including $G_m^2$ and $G_m^3$ (94). Vengris et al. (198) added specific gangliosides to an interferon resistant cell line and reconstituted its ability to bind interferon in a functional manner. Friedman et al. (57) showed that a ganglioside rich membrane extract was capable of binding human interferons. Kohn et al. (90) showed that TSH and cholera toxin, known to bind to membrane gangliosides, were capable of blocking interferon binding.

The present study was designed to determine if there is a functional association between the $G_m^1$-like antigenic determinant described in Part 1 and the ability of interferon to bind to NK cells and augment their cytolytic activity. As in Part 1, antibodies to $G_m^1$, both monovalent or multivalent, were used as an experimental probe to determine if such an association exists. It was thought that if these antibodies could block, or in some other way alter, the ability of interferon to bind to NK cells and augment the cell's response it would be indicative of a structural-functional association.
Materials and Methods

Cell Handling

Part 1 described the animals and human subjects used for this study as well as the protocol for the preparation of peripheral blood mononuclear cells (PBMCs) from venous blood.

MOLT-4 cells, a T cell lymphoblastoid cell line, were maintained in 75 cm² plastic tissue culture flasks containing RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and streptomycin (GIBCO). Cell cultures were passed three times weekly. The MOLT-4 cells were labeled with ⁵¹Chromium (⁵¹Cr New England Nuclear, Boston, Mass.) by the addition of 150 uCi of ⁵¹Cr to 1.0 to 1.5 X 10⁷ cells. After a 60 minute incubation at 37°C, the cells were diluted and centrifuged over a layer of Ficoll-Hypaque. Cells at the interface were washed 4 times and adjusted to the appropriate concentration.

Anti-C₄ Modulation of Interferon Augmentation of NK Cell Activity

After an overnight culture in RPMI-1640 containing 10% human serum, PBMCs were washed in Seligmann's balanced salt solution (SBSS) containing 0.5% human serum albumin (SBSS-HSA). Cells, 4 x 10⁶ in 500 ul were incubated for 30 minutes with the appropriate antisemur or SBSS for 30 minutes at 37°C and in 5% CO₂. Human fibroblast interferon (Calbiochem - Behring Corp., San Diego, CA), at 100 or 200 units, was then added to the cells and the incubation was continued for an
additional 60 to 90 minutes at 37°C in 5% CO₂. Unbound antibodies
and/or interferon were removed by washing twice with SBSS-HSA. The
washed cells were resuspended in RPMI-HS, counted, and assayed for
cytotoxicity against MOLT-4 target cells.

NK cell mediated cytolysis of ⁵¹Cr-labeled target cells was done in
accordance with the procedure of Opremcak et al. (128). To round bottom
microtiter plates, (Linbro, New Haven, Conn.) was added 50 ul of
⁵¹Cr-MOLT-4 cell (1x10⁵ in RPMI-HSA) and 200 ul of antibody and/or
interferon pretreated effector PBMCs at varying cell concentrations.
The pretreated effector PBMCs to target cell ratios used here were 5:1,
12.5:1, 25:1 and 50:1. The microtiter plates were centrifuged at
200 x g for 1 minute and incubated for 4 hours at 37°C. The assay was
terminated by a 5 minute 200 x g centrifugation. Triplicate supernatant
samples, 125 ul, were counted on a Beckman LS 7000 gamma counter.

Maximal release (MR) of ⁵¹Cr was obtained by pretreatment of target
cells with 1% Triton X 100, and spontaneous release (SR) was taken as
the counts per minute (cpm) recovered from supernatants of target cells
incubated in the absence of effector PBMCs.

The percent of specific ⁵¹Cr released was determined by the
formula:

\[
\% \text{ Specific Release} = \frac{\text{Exp. cpm} - \text{SR cpm}}{\text{MR cpm} - \text{SR cpm}} \times 100
\]
Antisera

Refer to Part 1 for the details of the production, purification and characterization of antibodies used in these experiments.

The Fab fragments of normal rabbit serum IgG (Cappel Laboratories, Inc., Cochransville, Pa.) and anti-\( \text{G}_{\text{M}1} 16P_4 \) were prepared as described by Stanworth and Turner (180). To the IgG antibodies in a 0.01 M phosphate buffer (pH 7.4) containing 0.01 M cysteine and 0.002 M EDTA, was added papain, (Sigma Chemical Co., St. Louis, Mo.) (1.0 mg papain per 100 mg IgG). This enzyme-antibody mixture was incubated for 16 hours at 37°C, and then it was in order to inactivate the enzyme dialyzed against 4 changes of distilled H_2O in a 48 hour period. The digest was additionally dialyzed against a 0.01 M sodium acetate buffer (pH 5.5) before applying it to a CM (carboxymethyl)-BioGel A (Bio-Rad Laboratories-Richmond, Ca) column (20 x 2.5 cm) equilibrated with 0.01 M acetate buffer (pH 5.5). The column was eluded with 200 ml of the column buffer and then a linear gradient from 0.01-0.9 M acetate buffer (pH 5.5) was applied with the aid of a LKB 11300 Ultragrad Gradient Mixer (LKB Instruments, Stockholm, Sweden). The first two peaks were pooled, back concentrated, and dialyzed against SBSS for 24 hours with 2 changes. SDS-PAGE electrophoresis yielded a single band with an approximate molecular weight of 23,000 daltons.
Results

PBMCs incubated with human fibroblast interferon (FIF) demonstrated a significant augmentation of NK cell cytolysis of MOLT-4 target cells (Figures 7, 8, 9, and 10). Augmentation varies from 9.0% to 20.0% reflecting both individual differences as well as differences in the effector-to-target cell ratio. Preincubation of PBMCs with varying protein concentrations of monovalent, Fab, antibodies to G\_1 results in a modulation of this augmentation. Modulation is a function of the Fab antibody fragment and not of the whole immunoglobulin molecule (data not shown). Similar results have been reported by Lingwood et al. (99) who were able to inhibit murine sarcoma virus induced transformation of 3T3 BALB/c cells with monovalent antibodies to G\_1 or to the Gap-a galactoprotein.

A total of five variables were examined in this series of experiments. They included: modulation vs. Fab antibody concentration, modulation vs. effect cell concentration, modulation vs. FIF concentration, NRS vs. anti-G\_1 Fab modulation and modulation due to preincubation conditions.

Fab antibody fragments, recognizing the G\_1-like antigenic determinant, exhibit either a negative or a positive modulation of FIF augmentation of NK cell activity. This capability varies with the protein concentration and is noted to occur irrespective of
irrespective of effector-to-target cell ratio or the amount of FIF present during the preincubation of PBMCs (Figures 7, 8, and 9). A comparison of the results in Figures 7 and 8 demonstrates that the degree of modulation exhibits individual variations. The amount of inhibition varies with the concentration of Fab protein. Results of Figure 8 indicate that these monovalent antibodies can also inhibit unaugmented NK cell activity. These results, however, are considered to be insignificant based on preliminary studies which showed Fab antibodies to GI to be incapable of modulating NK cell activity in the absence of interferon. Irrespective of other variables, the peak of positive modulation was noted as occurring within a very narrow range of protein concentrations. In the presence of 100 units of FIF, this peak occurred in the range of 0.6 to 0.8 mg of protein (Figure 7 and 8) which is increased to 1.0 mg when 200 units of FIF are used (Figure 9).

The degree of both positive and negative modulation changes relative to the effector-to-target cell ratio. The values of 5:1 and 12.5:1 were used in Figures 7, 8, and 9, based on the significant differences in unaugmented as compared to FIF augmented NK cell activity. The difference in these values decrease with increasing effector-to-target cell ratios (Figure 10). The peak of positive modulation becomes narrower at a 12.5:1 ratio, but it occurs within 0.2 mg of the value noted at 5:1. It was also noted that the degree of positive modulation was greater at 12.5:1 ratio as compared to a effector-to-target cell of 5:1. Changes in the suppression of FIF-NK cell augmentation occur with an increase in the effector-to-target ratio.
but the pattern is not consistent.

Incubation of PBMcs with Fab antibodies to $G_m^1$ in the presence of 200 units of FIF (Figure 9) as compared to 100 units (Figures 7 and 8) does not overcome the suppression of FIF augmentation of NK cell activity. The ability of the monovalent antibodies to increase the effector of FIF-NK cell augmentation is significantly decreased in the presence of 200 units of FIF as compared to that seen with 100 units. In addition, the peak of positive modulation is shifted to a higher protein concentration.

In order to demonstrate the specificity ofthe observed modulation by Fab fragments of antibodies to $G_m^1$, a series of parallel experiments were run using Fab fragments of NRS-IgG. The results of these experiments (Figures 8 and 9) indicate that the monovalent fragments of NRS-IgG can suppress FIF's augmentation of NK cell activity without demonstrating the concurrent positive modulation noted with anti-$G_m^1$ reactive Fab fragments. NRS may not be an appropriate control for these experiments due to the presence of antibodies reactive to human cell surface components (186). In addition, Nair and Schwartz (120) have just recently reported that normal murine and human serum can be inhibitory to their respective NK cell.

The results expressed in Figures 7, 8, and 9 were based on an initial 30 minute pretreatment of PBMcs followed directly, without washing, by an additional 60 to 90 minute incubation with FIF. In order to eliminate the possibility that the enhancement and/or suppression of FIF augmentation of NK cell activity by Fab antibodies was due to a
Fab-FIF complex, PBMCs were incubated overnight in the presence of 5.0 mg of Fab antibodies to G\textsuperscript{M1} and then thoroughly washed prior to the addition of FIF. The results of this experiment are seen in Figure 10. At this protein concentration only a suppression of FIF's ability to augment NK cell activity is noted. This experiment needs to be repeated with NRS-Fab antibodies as well as with lower concentrations of the G\textsuperscript{M1} reactive Fab fragments. Yet, the results noted here do eliminate the possibility that a Fab-FIF complex induces the results seen and further indicates the Fab antibodies are blocking the binding of FIF to the NK cell surface.
Discussion

The results of this study demonstrate that Fab, monovalent antibodies to the G\textsubscript{M1}-like antigenic determinant can modulate the augmentation of human NK cell activity by fibroblast interferon (FIF). This modulation is both positive and negative and is dose dependent relative to the concentrations of both Fab-anti G\textsubscript{M1} and FIF. The degree of modulation is dependent on the effector to target cell ratio used in the assay. Suppression of this augmentation occurs with NRS-Fab antibodies, but there is no enhancement as noted with Fab\textsuperscript{'}anti-G\textsubscript{M1}. There are several possible explanations for this complex bimodal modulation, but no one explanation can totally account for the findings presented here.

An immediate assumption would be that the negative modulation, or suppression, is actually an artifact of the experimental protocol and that only enhancement is of a significant nature. This explanation would necessitate the fact that only Fab antibody fragments, but not whole antibody molecules, could bind to the surface and block the action of FIF in a nonspecific manner. Support for this explanation comes from the results of Pitha et al. (136). They used an antiserum to the surface of human fibroblast in an attempt to block interferon binding. Pitha and coworkers's failure to inhibit cell surface binding of interferon with whole antibody molecules is in contrast to the results.
of Revel et al. (145). These latter authors were able to inhibit an interferon induction of an antiviral state by pretreating the cells with whole antibodies to a cell membrane component specified by chromosome 21. Also in conflict with the assumption that enhancement is significant while suppression is an artifact are the results in Figure 7 where only an enhancement of the augmentation is seen at an effector to target cell ratio 5:1 and inhibition only occurs at the highest protein concentration when the 5:1 ratio is increased to 12.5:1.

The binding of Fab anti-\(G_{M1}\) to the cell responding to FIF could facilitate one or more of the following results: a direct inhibition of FIF binding to one of its receptor components, an inhibition of a signal transduction following FIF binding, or an initiation of a "new" signal being transmitted into the cytoplasm. The first two possibilities would result from a molecular alteration of the membrane receptor for FIF; whereas, the third would result in a metabolic change in the FIF responsive cell. As presented here, the results of this study do not clearly distinguish among these possible explanations.

The membrane receptor for interferon consists of both a glycoprotein and one or more ganglioside molecules (57,66). The exact gangliosides comprising the receptor are unknown due to the fact that several are capable of binding interferon (94,198). The ganglioside may or may not form part of the binding site. These molecules function in signal transduction, but are considered to exhibit both nonspecificity and low affinity for interferon binding (15,24,66, 90). If the ganglioside molecule actually forms part of the molecular conformation
of the interferon receptor (66) then we would expect to see a competitive inhibition of FIF binding by the Fab anti-\(G_{M1}\). What is seen is a peak of enhancement which occurs within a very narrow range of antibody concentration. Direct competitive inhibition of FIF binding by Fab anti-\(G_{M1}\) can not explain the continued suppression of augmentation at antibody concentrations less 1.0 mg when the concentration of FIF is doubled (Figure 9).

Gangliosides are essential for the transmission of one or more signals resulting from interferon binding to the cell surface (60,90). This function is dependent on their lateral mobility in the plane of the membrane (12). Evidence for such mobility comes from the results of Friedman (56) and Chany et al. (24). Friedman showed that the binding of interferon can occur at 4°C, but that the induction of the antiviral state does not occur until 37°C. Chany and coworkers demonstrated that an impairment of interferon induction of an antiviral state occurs when the cytoskeletal elements are disrupted. The Fab anti-\(G_{M1}\) could bind to the ganglioside component and thus inhibit its ability to transmit a signal induced by interferon binding. These antibody fragments are not able to crosslink the gangliosides, and therefore one would only expect microclusters, rather than large aggregates, following Fab anti-\(G_{M1}\) binding to the cell.

Interferon binding to the cell surface induces a physical alteration in the surface exposure of various glycoconjugates (66). These changes may give rise to signal transmission. Lingwood et al. (99) have shown that Fab anti-\(G_{M1}\) is capable of "freezing" the membrane
and preventing alterations in ganglioside expression arising after viral transformation. The Fab anti-\(G_{M1}\) may induce such a freeze and prevent any alteration in the surface of cells responding to interferon. The cell in this case may be a pre-NK cell. Minato et al. (111) have shown that in recruitment of murine pre-NK cells interferon induces the expression of the Ly5 antigenic determinant. Thus, Fab anti-\(G_{M1}\) may prevent the interferon directed differentiation of pre-NK cells. The results of Fuse et al. (61) tend to refute this possibility. They showed that binding of the B subunit of cholera toxin to human PBMCs does not alter the ability of interferon to augment NK cell activity, whereas the binding of whole cholera toxin does. Lingwood and Hakomori (98) have shown that Fab anti-\(G_{M1}\) has a bimodal effect on ganglioside expression on pretreated cells. They showed that net \(G_{M3}\) and \(G_{D1a}\) synthesis was stimulated initially, but that with time it was inhibited. This bimodal response is time and not antibody concentration related as seen in this study. These results, however, do demonstrate the ability of monovalent antibodies to gangliosides to alter the cell surface, i.e., "freeze" it. An alteration in the membrane surface may interfere with simple signal transduction following interferon binding. This would not explain the need for higher Fab protein concentrations to induce enhancement in the presence of 200 units rather than 100 units of FIF (Figures 7, 8 and 9); whereas, such a mechanism would explain the results seen in Figure 10.

A further complication to the interpretation of these results was an observation that Fab anti-\(G_{M1}\) is unable to augment or suppress NK
in the absence of FIF or cellular response to FIF. These were initial studies and must be repeated, but they indicate that FIF must be present and the NK cells responsive to it in order for the Fab anti-\(G_{M1}\) to exert its effect. It can be concluded then that Fab anti-\(G_{M1}\) does not block FIF binding, but that it modulates the cell's response to FIF. The bioactivity of interferon is a result of physical changes in the cell surface, changes in ion fluxes across the membrane, and activation of adenyl cyclase (66). Of these three, an alteration in cyclic nucleotide levels would best explain the bimodal nature of Fab anti-\(G_{M1}\) effect of FIF's augmentation of NK cell activity.

Intracellular levels of cyclic nucleotides modulated NK's cytolytic activity, both in vitro (61,148) and in vivo (22). NK cell activity has been reported to be enhanced by increasing cytoplasmic level of cGMP and decreased by elevated cAMP levels (61,148). Additionally, interferon augmentation of NK cell activity has been similarly reported to be enhanced by increasing cGMP and suppressed by increasing cAMP in the cytoplasm (61). This then is circumstantial evidence that interferon's effect on NK cells is to modulate their cyclic nucleotide levels. Cholera toxin can induce a suppression of FIF augmentation of NK cell activity prior to or after interferon is added to the cells (61). In conflict with the above results is the study by Meldolesi et al. (108) who reported that increased cAMP precedes an interferon induced antiviral state; whereas, several authors (43,58,60) have shown that cholera toxin inhibits this induction in a dose dependent manner.
The binding of Fab anti-\( G_M^1 \) could induce the formation of microclusters of the \( G_M^1 \)-like antigenic determinant, even though the Fab molecules cannot crosslink the antigenic determinants and subsequently generate a "new" signal. Such lectin induced lipid domains have been discussed by Curtain et al. (36). This "new" signal is considered to be cAMP and not cGMP. Preliminary experiments have indicated that these monovalent antibodies are capable of stimulating adenyl cyclase activity in PBMCs, but this work needs to be expanded upon and refined before a definitive statement can be made. Holmgren and Lindholm (78) reviewed the fact that intracellular cAMP levels can either stimulate or suppress an immune response and that it is determined by the concentration of the lectin, in their case cholera toxin, binding to the cell surface.

The peak of enhancement is considered here to result from an optimal intracellular concentration of cAMP being generated by the binding of both Fab anti-\( G_M^1 \) and FIF. Meldolesi et al. (108) have similarly shown that the cAMP levels generated by interferon and 3-isobutyl-1-methyl xanthine are additive for inducing an antiviral state in murine Ly cells, but not in cells which lack gangliosides. No peak is associated with the binding of NRS Fab-fragments, but the suppression that they exert most likely arises from nonspecific binding to the cell surface which will reduce the interferon induced signal transmission. At protein concentrations greater than the optimum at the peak there is too much stimulation of adenyl cyclase and thus suppression of FIF augmented NK cell activity. This latter explanation would correspond to the results of Fuse et al. (61), and would also account for the results shown on Figure 10 where PBMCs were incubated
overnight in the presence of 5.0 mg of Fab anti-\text{G}_{\text{M1}}. These latter results indicate that the suppression is not reversible. This is in accord with the results reported by Grollman et al. (66) that inhibition of interferon activity by cholera toxin is irreversible. The decrease in augmentation noted in Figure 7 as occurring at less than .6 mg or .8 mg of Fab results from a decrease in the optimum value of cAMP for NK cell cytolysis. This does not explain the results noted in Figure 8 where Fab concentrations less than the optimum induce a marked suppression in NK cell activity. What these graphs (Figure 7 and 8) do depict are the individual differences seen in NK cell response to interferon augmentation.

Another possible explanation is that at the lower protein concentrations there is an over stimulation of adenyl cyclase and thus a suppression of the augmentation. The peak would again result from an optimal concentration of cAMP. The marked inhibition of augmentation at higher concentrations would result from an antibody induced alteration in the membrane blocking interferon's induction of a signal being generated. This would explain the results on Figures 7, 8, and 10. In this latter case the membrane alteration would be irreversible.

What these explanations don't necessarily account for are the results on Figure 9. The peak represents an optimal intracellular cAMP level, but in the presence of 200 units of FIF there may be a competition for adenyl cyclases and thus there is a smaller peak. The inhibition at 2.0 mg of Fab anti-\text{G}_{\text{M1}} could arise from membrane alterations. But why is there a peak at 1.0 mg, when this was previously shown to been inhibitory (Figures 7 and 8)? This question
may be answered by the possibility that both FIF and Fab anti-GL compete for a common site on or in the membrane. The suppression of augmentation could again be due to an over stimulation of adenyl cyclase at these protein concentrations.

This data is complex and its interpretation is difficult. Many more experiments need to be performed and more variables accounted for. This should provide a very interesting area of research for the next few years.
FIGURE 7: Anti-\(G_{M1}\) Modulation of Interferon Augmentation of NK Cell Activity. Adult peripheral blood mononuclear cells were pretreated with varying concentrations of the Fab fragment of 16P4 IgG anti-\(G_{M1}\) serum, followed by 100 units of human fibroblast interferon (FIF). Controls: NK cell activity alone (NK) and NK cell activity augmented with FIF (NK + FIF).
FIGURE 7: ANTI-GM1 MODULATION OF INTERFERON AUGMENTATION OF NK CELL ACTIVITY
FIGURE 8: Comparison of the Ability of Anti-GM1 and NRS to Modulate Interferon Augmentation of NK Cell Activity. Adult peripheral blood mononuclear cells were pretreated with varying concentrations of the Fab fragments of 16P4 IgG (●) or NRS IgG (○), followed by 100 units of human fibroblast interferon (FIF). Controls: NK cell activity alone (NK) and NK cell activity augmented with FIF (NK + FIF).
FIGURE 8: COMPARISON OF THE ABILITY OF ANTI-\(\text{G}_{\text{M}1}\) AND NRS TO MODULATE INTERFERON AUGMENTATION OF NK CELL ACTIVITY.
FIGURE 9: Anti-G\textsubscript{M1} Modulation of NK Cell Activity in the Presence of 200 Units of Interferon. Adult peripheral blood mononuclear cells were pretreated with varying concentrations of the Fab fragments of 16P\textsubscript{4} IgG (●—●) or NRS IgG (○—○), followed by 200 units of human fibroblast interferon (FIF). Controls: NK cell activity alone (NK) and NK cell activity augmented with FIF (NK + FIF).
FIGURE 9: ANTI-\( \text{G}_{\text{M1}} \) MODULATION OF NK CELL ACTIVITY IN THE PRESENCE OF 200 UNITS OF INTERFERON
FIGURE 10: Modulation of NK Cell Activity by FIF Following an Overnight Incubation with Fab Anti-G_{M1}. Adult peripheral blood mononuclear cells were incubated overnight in the presence of 5.0 mg of Fab 16P_{4} IgG. After washing the cells were treated with 100 units of human fibroblast interferon (○—○). Controls: NK cell activity alone (●——●) and NK cell activity augmented with FIF (□——□).
FIGURE 10: MODULATION OF NK CELL ACTIVITY BY FIF FOLLOWING AN OVERNIGHT INCUBATION WITH Fab ANTI-GM1
Preparation of Rabbit Antisera Gangliosides

Numerous immunization protocols have been used in the last 15 years to raise antibodies to gangliosides. Rapport et al. (140) showed by comparing many of these techniques, that there is always a variation in a given animal's response to gangliosides as immunogens. Variability arises from the fact gangliosides are not very immunogenic and that their physical orientation in the aqueous phase greatly affects their immunogenicity (4). Several procedures have been employed to maximize a given animal's response to injections of immunogenic preparations of gangliosides.

Gangliosides are considered to be haptens; therefore, they require a protein carrier. This protein carrier should be a heterologous protein, but there is no need for them to be covalently linked (37). Bovine serum albumin, methylated or not, and/or complete Freund's adjuvant are the carriers of choice, and are usually used together during initial immunization. Zalc et al. (214) concluded that methylated, rather than unmethylated, bovine albumin is a better carrier for acidic glycolipids, which includes gangliosides. This is due to the methylated albumin's basicity which allows for a strong electrostatic complex to be formed with gangliosides (4). Marcus and Kundu (106) have recently reported success in antibody production to gangliosides.
covalently linked to various carrier proteins.

Gangliosides alone in an aqueous media will form micelles. This is not the optimal manner in which gangliosides can be presented to the immune system. Ohsawa and Nagai (127) incorporated gangliosides, cholesterol and egg lecithin together into liposomes and obtained significantly higher titers than with injections of micelles alone. The use of auxiliary lipids creates the optimal conditions for hapten orientation resulting in a multivalent form (4). This is the basis for using auxiliary lipids in the various techniques used to characterize antibodies to gangliosides. In agreement with the findings of Rapport et al. (140) and Taketomi and Uemura (186), I found that antisera titer is independent of whether liposomes or micelles of the ganglioside are used, but is dependent on the individual rabbit used.

In addition to variations in carrier proteins used, and the physical form of the gangliosides, there is yet no standard protocol for route and form of antigen injections. Most studies have combined the antigen with a heterologous protein and formed an emulsion with complete Freund's adjuvant. Foot pad and intradermal injections are the most common route for administering the initial injection. Intravenous and intraperitoneal injections have been used with success for both initial and/or booster injections (92,133,140,186). Booster injections vary in time and route of injection.
APPENDIX B

Purification of Antibodies to Gangliosides

The use of anti-ganglioside sera to detect these antigens on the cell surface is complicated by the presence of: antibodies to BSA, of naturally occurring antibodies to cell membrane components and relatively small amounts of ganglioside-specific antibodies. These problems have been minimized by the use of various ganglioside-immuno-adsorbents which function to purify and concentrate antibodies specific for gangliosides.

In the technique of Coulon-Morelec (29), cholesterol crystals serve as the insoluble solid support. The glycolipid hapten, alone or in combination with lecithin, is adsorbed to the crystal's surface. The resulting immunoradsorbent is mixed with the antiserum. Following centrifugation the adsorbed antibodies are removed with a 2.5M NaCl-0.01M BaCl₂ solution. The dissociated antibody-antigen complex is further separated with a 0.5% lecithin-Ether solution. The antibodies remain in the aqueous phase while the lipids enter the ether phase. This technique is capable of removing both IgG and IgM bound antibody molecules and the efficiency is reported to be 90%.

Glycolipid containing liposomes represent another form of solid support immunoadsorbent (5). The antiserum is mixed with the liposomes in excess and allowed to incubate for up to 60 minutes. The adsorbed
The adsorbed antibody was removed by either extraction with 0.15 NaCl in choloform or with NaI (1.0M) and the liposomes and antibody separated by ultracentrifugation. The authors reported a 175 to 3800 fold purification. The major disadvantage is the fact that antibody binding is inversely related to the phospholipid fatty acyl chain.

Marcus (105) incorporated a mixed micelle of ganglioside-lecithin-cholesterol into a 10% acrylamide solution which was then polymerized. The polymerized gel was homogenized, washed, and mixed with a equal volume of Sephadex G-25 to form an immunoadsorbent column. This technique results in 80-90% of the micelles being incorporated into the gel. A serum sample was added and the flow maintained at a slow rate. Bound antibodies are removed with 2M potassium thiocyanate (KSCN) and dialyzed.

Three groups have prepared immunoadsorbent columns where the ganglioside are covalently linked to glass beads (95,192,211) or aminopropyl silica gel (93). With the use of glass beads, the double bond in the sphingosine (Figure 1) is converted to a carboxyl group and reacted with glass beads containing free amino groups. A similar amide bond is formed between the carboxyl group of a sialic acid residue and silica gel derivatized to aminopropyl silica gel. Antibodies to gangliosides are removed by using a flow rate less than gravity. Bound antibodies are removed with a chaotropic agent, usually SCN⁻ ions. Kundu and Roy (93) reported that up to three times as much purified antibody was obtained using this form of affinity column as compared to gangliosides incorporated into polyacrylamide gel.
A major drawback of immunoadsorbent techniques is the specific or nonspecific binding of nonimmunoglobulin serum proteins to the insolubilized antigen. Alving and Richards (5) reported nonimmunoglobulin contamination of liposome bound serum proteins, and that the amount and type varied with the antiserum sample. Kundu and Roy (93) reported no nonimmunoglobulin serum protein binding to their immunoadsorbent column. This is in contrast to our findings of two or more contaminants binding to the Gm1-aminopropyl-silica gel columns (Plate 1). Important also is the fact that the eluted antibodies in each case lacked activity to BSA, and this indicates that there is no nonspecific binding to these columns.
APPENDIX C

Determination of Antibody Activity to Gangliosides

Numerous immunochemical techniques have been used to detect and characterize antibodies to gangliosides (Table 12). Despite differences in sensitivity, these techniques share the common feature that gangliosides are first mixed with cholesterol and lecithin prior to reacting the antigen with an antiserum. Mixed lipid micelles and/or liposomes of antigen are prepared by: adding the lipids together in a set ratio by weights, drying them down under a stream of N₂, after which the mixed lipid micelles are resuspended in buffer or solvent and dispersed by sonication. (Mixed lipid micelles in a solvent will keep for months until diluted with buffer and resonicated). The presence of cholesterol and lecithin, as auxiliary lipids, results in the carbohydrate residues of the gangliosides being presented to antibodies, in an aqueous environment, in such a way as to maximize their molecular interaction (4,105,125).

These techniques vary in their sensitivity to detect antibodies to gangliosides. Different conclusions can be reached depending on the technique being used (4). Detection of antibodies by double diffusion is relatively quick and simple; however, it is the least sensitive of the methods listed and often leads to "spurious reactions" (105). Complement fixation (CF) and complement-mediated lysis of antigen
containing liposomes is much more sensitive than double diffusion in agar (105). CF can yield semiquantitative data indicating the amount of antibodies present in the serum sample (105) and can yield information as to antibody-antigen interaction (140). This assay also allows for determination of antiserum specificity and antigen epitope studies. Disadvantages center around the sensitivity of the assay to the anti-complementary activity of rabbit serum (106) and gangliosides (4). This latter fact is minimized, but not eliminated, by the presence of auxiliary lipids (4). Marcus and Kundu (106) concluded that the CF technique is not useful for antisera with titers less than 1:64. Results can also vary due to the temperature and period of incubation used. Maximal CF activity for IgM is obtained by a 60 minute incubation at 37°C, which varies from the overnight incubation at 4°C needed for optimal complement fixation by IgG (105).

Complement-mediated antibody lysis of liposomes was designed by Kinsky and coworkers (88) as a model analogous to natural cell membranes. Liposome membranes contain gangliosides, cholesterol, lecithin and dicetyl phosphate, while the aqueous compartment contains a marker substance. Numerous markers have been used including glucose (88), various enzymes (85), a fluorescent quencher compounds (178), and radioisotopes (174). The antibody activity, in the presence of complement, is measured as a function of marker release. Liposomes can be used for the detection of antibodies and the determination of their specificity. Disadvantages of this technique include maintenance of optimal conditions for enzyme activity, and the need for column
chromatography following lysis of radioisotope containing liposomes. In addition, not all laboratories may be equipped for liposome production.

Of these three techniques, I have found more reproducible results were obtained with a modification of a micro-complement fixation technique (109). This test is fairly rapid and all variables: buffer, complement, antiserum, antigen, and sheep erythrocytes, can be monitored with adequate controls. The preparation of antigen micelles is much quicker than of liposomes and requires much less expense in supplies and equipment. Titers of 1:10 or 1:20 were obtained with liposome lysis as compared to 1:500 or 1:1000 with the same sample using a complement fixation assay.

Passive hemagglutination, using ganglioside coated erythrocytes, is reviewed by Marcus (105) as a good method to detect IgM antibodies to gangliosides. He further concluded that the IgG antibodies may go to totally undetected and that results may be further complicated by naturally occurring antibodies to the indicator erythrocytes. These disadvantages are minimized by the use of antigen containing liposomes (59,195). Uemura et al. (195) concluded that liposome agglutination effectively detected IgG antibodies, while IgM molecules were better detected by liposome lysis techniques. Fry et al. (59) concluded that liposome agglutination was a rapid method for detection of antiglycolipid antibodies. Rapid precipitation techniques using liposomes (59) and micelles (83) have recently been used for serological studies of antisera to glycolipids.
The use of radioimmunoassays for the detection of antibodies to gangliosides is just now being developed. An RIA is the most sensitive of all immunochemical techniques, and offers the advantage of measuring the "formation of an antigen-antibody complex, rather than a secondary event such as precipitation or complement fixation" (106).
TABLE 15

Immunochemical Techniques for Detection of Antibodies to Gangliosides and other Glycolipids.

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