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THY-1 GLYCOLIPID MODULATION
OF THE
IN VIVO IMMUNE RESPONSE

By

Alvin A. Gabrielsen, Jr.

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ABSTRACT

THY-1 GLYCOLIPID MODULATION OF THE *IN VIVO* IMMUNE RESPONSE

By

Alvin A. Gabrielsen, Jr.

The *in vivo* functional role of the Thy-1 alloantigen, known to be shed from antigenically stimulated T lymphocytes, has been investigated in this report. Mice were challenged i.v. with sheep erythrocytes (SRBC) followed 24 to 48 hours later by i.v. injections of gangliosides containing Thy-1 activity (formulated into cholesterol:lecithin liposomes by low frequency sonication at 60 Hz). Both suppressed and enhanced anti-SRBC plaque-forming-cell (PFC) responses were observed in the Thy-1-treated mice in a hemolytic plaque assay. $B10C3F_1$ mice (Thy-1.2) receiving 5×10^8 SRBC demonstrated suppressed IgM PFC responses of 60-81% when treated with CBA G_{M1} ganglioside (containing Thy-1.2). CBA G_{D1b} (with some Thy-1.2 activity) and AKR G_{M1} (containing Thy-1.1) gangliosides also depressed PFC responses to a lesser extent in $B10C3F_1$ mice. Indirect responses in this system were suppressed by as much as 97%. Exposing mice to gangliosides with Thy-1 glycolipid prior to SRBC immunization did not increase the degree of suppression.

$B6C3F_1$ mice, given 5×10^7 SRBC, followed by Thy-1 treatment

demonstrated enhancement of the primary (IgM) immune response. (IgG responses by the day of assay were seldom observed using the above SRBC dose.) This was observed in mice treated with Thy-1-containing liposomes formulated by high frequency (HF; 20,000 Hz) or low frequency (LF; 60 Hz) sonication. Enhancement with HF and LF liposomes was 38% and 100% over controls, respectively, with 6 µg doses of G_{M1} (plus Thy-1) given at 24 and 48 hours after antigen. Sixteen micrograms of G_{M1} incorporated into HF liposomes were able to increase IgM PFC by 100% over controls when given at 24 and 48 hours, while administration of 6 µg doses at 6 and 30 hours after antigen did not significantly increase the degree of enhancement seen at that dose given at 24 and 48 hours. In the absence of auxillary lipids (cholesterol and lecithin), G_{M1} (plus Thy-1) was found to exert no effect on the PFC response.

The kinetics of Thy-1-dependent enhancement revealed that the effect was measurable in the direct response by day 6 after antigen injection as IgM PFC declined from peak values. In this study, a significant IgG response was measured by day 5 for Thy-1-treated mice. No significant control IgG PFC were observed until day 7, and then at only 10% of the level of the Thy-1-treated group. The relative enhancement in IgG PFC declined as the responses of both groups increased with time, but was still significantly higher by day 10 in the group treated with Thy-1. These results suggested that Thy-1 may suppress or enhance the humoral immune response in mice. Since $B10C3F_1$ mice treated with Thy-1 glycolipid displayed suppression of the PFC response, while enhancement of the response was evident in $B6C3F_1$ mice under similar conditions, the effect was probably partially dependent on the mouse genotype.

The kinetics of the enhancement effect imply a more important role of Thy-1 in the secondary response than the primary response.

DEDICATED TO

KARLEEN AND JACOB

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LIST OF ABBREVIATIONS

ABShu	anti-human brain antiserum
ABSm	anti-mouse brain antiserum
AEF	allogenic effect factor
ATS	anti-thymocyte serum
BGG	bovine gamaglobulin
BRBC	burro red blood cells
BSA	bovine serum albumin
cer	ceramide
C:L	cholesterol : lecithin
C:M	chloroform : methanol
Con A	Concanavalin A
CRBC	chicken red blood cells
CT	cholera toxin
DNP	dinitrophenol
Gal	galactosyl
GalNAc	N-acetylgalactosaminyl
GAT	L-glutamic acid^{60} - L - alanine^{30} - L - lysine^{10}
G_{D1a}	Gal(NANA)-GalNAc-Gal(NANA)-Glc-cer
G_{D1b}	Gal-GalNAc-Gal(NANA-NANA)-Glc-cer
Glc	glucosyl
G_{M1}	Gal-GalNAc-Gal(NANA)-Glc-cer
HF	high frequency (20,000 Hz)

Ig	immunoglobulin
i.v.	intravenously
KLH	keyhole limpet hemocyanin
LF	low frequency (60 Hz)
LPS	lipopolysaccharide
MBSA	methylated bovine serum albumin
MEM	minimal essential medium
MIF	migration inhibition factor
MuMTV	mouse mammary tumor virus
NANA	N-acetylneuraminyl (sialic acid)
NP-40	Nonidet P-40
OVA	ovalbumin
PBS	phosphate buffer saline
PFC	plaque forming cells
PHA	phytohemagglutinin
POL	polymerized flagella
PRBC	pigeon red blood cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRS	soluble immune response suppressor
SRBC	sheep red blood cells
(T,G)-A--L	poly (tyr-Glu)-poly DL Ala--poly-Lys
TLC	thin layer chromatography
TNP	trinitrophenol
TT	tetnus toxin

INTRODUCTION

Since its discovery, the Thy-1 alloantigen has become a convenient cell membrane marker for the study of thymus-derived lymphocytes (T cells) in the murine system. It has provided a basis for identification of the cell types and the cellular interactions that appear to be necessary in order for a host to mount a successful protective response to a foreign antigen.

The following literature review has been organized to provide background information on the Thy-1 antigen, summarizing the details of its discovery, the attempts that have been made to characterize it, and the _in vitro_ studies that have suggested a possible functional role for Thy-1 in regulation of the immune response. In addition, the role of Thy-1 bearing cells in regulation of the humoral immune response has been briefly reviewed here. This was done to put the functional role of Thy-1 in perspective with the multitude of regulatory factors derived from T cells upon antigenic stimulation.

The ultimate test of an _in vitro_ phenomenon is the demonstration of its _in vivo_ correlate. _In vitro_ studies showed that a glycolipid with Thy-1 activity exerted a temporary suppressive effect upon the antibody response to a given antigen, followed by an enhanced response to that antigen. The present study was undertaken to investigate the possibility that the Thy-1 glycolipid might play a similar role _in vivo_. Initial experiments looked at the effect of Thy-1 on the development of antibody-producing cells in the spleen in a regimen of antigen and Thy-1 administration based on the *in vitro* studies. Variations in the antigen and Thy-1 administration schedules were also investigated for their effects on antibody production. The importance of the combination of Thy-1 glycolipid with auxillary lipids for modulatory activity, and the nature of the resultant liposomes was also studied. Finally, the kinetics of the effect of the Thy-1 glycolipid on the primary and secondary antibody responses were investigated.

The results of the above studies are discussed in terms of a model of immune regulation proposed from the earlier *in vitro* studies. The role of the auxillary lipids and liposomes into which Thy-1 was incorporated for this study are discussed in relation to the method employed for assessing the *in vivo* effect of Thy-1.

LITERATURE REVIEW

Thy-1 Alloantigen

Introduction

Reif and Allen (1) first observed in 1963 that multiple injections of AKR mouse thymic lymphocytes into C3H mice elicited a strong cytolytic response against AKR thymocytes, despite H-2 histocompatibility (1). Further investigation, using the reverse procedure, revealed the existance of an alloantigen in C3H mice (2). These antigens have been designated Thy-1.1 and Thy-1.2 (previously Θ-AKR and Θ C3H, respectively - see ref. 2 and 3). Anti-Thy-1.1 and anti-Thy-1.2 alloantisera were prepared and tested against a large variety of mouse strains (2,4). Results showed that most inbred mouse strains carried the Thy-1.2 alloantigen on their thymocyte membranes, while only a few strains demonstrated Thy-1.1 specificity (2). All strains of mice so far studied, carry one or the other of these two allelic forms, which are coded for on chromosome nine of the mouse (5).

Distribution of Thy-1.

Reif and Allen (6) were unable to demonstrate significant amounts of the Thy-1 antigen in a variety of mouse tissues examined. Other murine tissues were tested for their abilities to absorb out the anti-Thy-1 activity of the alloantisera and little or none was found on bone marrow cells, in neonatal brain, appendix, lung, liver, skeletal muscle, kidney, and testes (2). Thy-1.1 and Thy-1.2 were found to increase from low levels in neonatal mouse brain and lymphoid tissues to maximum levels by 5 to 6 weeks of age (6,7,8).

Since the discovery of Thy-1 in the mouse, it has been identified in a number of nonlymphoid tissues (6,9-15), on T lymphocytes in various lymphoid organs (7-9,16-18), on several lymphoblastoid cell lines (2,21), and in species other than the mouse (14,22-28).

In the mouse, Thy-1 activity has been demonstrated in peripheral nervous tissue (2) and brain tissue (2,6,9-11), epidermal cells (12), normal and neoplastic mammary tissue (13), fibroblasts (14), and thymus epithelium (15). Thy-1-bearing lymphocytes are found in varying relative quantities in the thymus (7,8,16), thoracic duct lymph (7), lymph nodes (7,9,16), spleen (7,16), peritoneal cavity (7), Peyer's patches (7), and peripheral blood (17,18). Rapid appearance of Thy-1 positive lymphocytes in murine bone marrow after treatment with thymic factor (19), _Vibreo cholerae_ neuraminidase (19), or thymopoetin (20), suggests the presence of precursor T lymphocytes in the bone marrow carrying sequestered Thy-1 antigen in their plasma membranes.

In their early studies, Reif and Allen (2) discovered the presence of Thy-1 on the surface of many leukemias, distinct from other known characteristic tumor antigens. Zwerner and Acton (21) identified further lymphoblastoid cell lines carrying either the Thy-1.1 or Thy-1.2 alloantigen.

The presence of a Thy-1-like antigen in rats was reported in

1972 by Douglas (22). Absorption and direct complement-dependent thymocytotoxicity studies demonstrated a strong cross reactivity with the Thy-1.1 antigen of the mouse, while little Thy-1.2 activity was observed (22). Further investigation has revealed that Thy-1.1 is found in rat brain, thymic tissue (23), and on rat fibroblasts (14), comparable to the mouse system, while little is expressed on peripheral T lymphocytes (23). Thy-1.1 is abundant on rat bone marrow cells, being expressed on perhaps as many as 50 per cent of cells tested (24). Surface immunoglobulin, a B lymphocyte marker, is present on about 25 per cent of the Thy-1.1 positive rat bone marrow cells, suggesting that Thy-1 is not unique to "thymus-derived" lymphocytes in the rat (24).

Arndt et al. (25), investigating human tissue, reported anti-mouse Thy-1.2 activity in rabbit anti-human brain antisera (ABShu). The ABShu cytotoxicity to CBA (Thy-1.2) thymocytes could be absorbed out with human, rat, and CBA mouse brain. Conversely, anti-mouse brain antisera (ABSm) activity could only be removed by absorption with mouse brain. Cocapping experiments using mouse anti-Thy-1.2 and ABShu antisera, followed by fluorescent labeled goat anti-mouse globulin and goat anti-rabbit globulin, demonstrated the presence of two closely linked antigenic determinants for Thy-1, one of which was proposed to be species-specific, and the other nonspecies-specific (25). The nonspecies-specific determinant, previously described in human and mouse brain (25), was then found to be shared by human thymus (26), while the species-specific determinant could also be identified on human thymus, peripheral blood lymphyocytes, and bone marrow cells (26).

Isolation and Characterization of Thy-1 Cell Surface Antigen

Owing to a variety of techniques employed to isolate and characterize the Thy-1 antigen, the exact physiochemical nature of this moiety is still somewhat in question. Researchers have attempted to isolate Thy-1 from mouse brain tissue (9,29-31), T cells and/or thymocytes (4,29,30,32-36), lymphoblastoid lines (35,37-39), rat thymocytes (27,28), and rat brain (28). These tissues have been subjected to a variety of techniques designed to purify and identify Thy-1. Common procedures include detergent solubilization (27,31,35,36), followed by immunoprecipitation (31,35) or gel filtration and affinity chromatography (27,36). Thy-1 has been radiolabeled by ^{125}I (31,34) and ^{3}H amino acids and carbohydrates, followed by immunoprecipitation (31). It has been extracted by organic solvents after physical disruption of cells (30,31), or been subjected to enzyme solubilization followed by organic solvent extraction (38). Further purification steps have involved density gradient (31) and zonal gradient centrifugation (9), thin layer chromatography (30,40), isoelectric focusing (29), and separation by electrophoretic mobility (27,28,34-36). Biological activity before, during, and after combinations of the above techniques has been monitored by inhibition of thymocytotoxicity of heterologous and alloantisera to Thy-1 (30,38,41), ability to induce specific anti-Thy-1.1 or anti-Thy-1.2 activity in spleen cell cultures (42), and precipitability by specific anti-Thy-1 antisera (31,34,35).

In keeping with the various methods used to determine the nature of Thy-1, different conclusions have also been drawn. Thy-1 has been characterized as a protein (34,38), a lipoprotein (4,29), a glycoprotein (9,27,28,36,37,41), or a glycolipid (30,31,33,40,43,44).

Protein nature of Thy-1. Lactoperoxidase radioiodination of thymocyte cell surface proteins, followed by extraction with 10 M urea plus 1.5 M acetic acid, was the method employed by Atwell et al. (34), to begin Thy-1 isolation. Thy-1 was then immunoprecipitated in a double antibody system reacting mouse anti-Thy-1 antiserum with the thymocyte extract, followed by excess rabbit anti-mouse Ig. The precipitate was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) which suggested that Thy-1 activity resided in a protein moiety of 60,000 molecular weight.

Kucich et al. (38) found that the supernatant of crude papain-solubilized S-49.1-TB.2.3. murine lymphoblastoid cells had anti-Thy-1.2 inhibiting properties as measured by a ^{51}Cr-release cytotoxicity inhibition assay. Further digestion by crude papain diminished Thy-1.2 activity. Similar results with crystallized papain excluded the possibility that glycosidases or lipases were responsible for the decreased activity. Prolonged exposure to proteases destroyed the Thy-1.2 activity as did extraction of the supernatant by chloroform and methanol. This evidence was interpreted to suggest that Thy-1 was protein in nature (38).

Although evidence of the protein nature of Thy-1 had been presented, the possibility of carbohydrate or lipid involvement had not been ruled out.

Lipoprotein nature of Thy-1. Possible lipid involvement in Thy-1 activity was first suggested by Reif and Allen (4) in a study of the nature of the Thy-1.2 antigen present on C3H thymocytes. Solubilization of thymocytes was followed by dialysis and centrifugation at 35,000 x g. On the basis of unit nitrogen content, the microsomal fraction of the thymus homogenate was 19 times more potent than whole homogenate when tested for absorption of AKR alloantiserum against C3H thymocytes. This nondialyzable activity was not recovered after treatment with chloroform: methanol (2:1) and other lipid solvents. These results were interpreted as indicating that this membrane-associated molecule was lipoprotein in nature (4).

Arndt et al. (29) isolated Thy-1 from mouse brain and thymocytes and studied it in an anti-Thy-1 cytotoxicity inhibition system. Brain or thymocytes were first solubilized in the nonionic detergent Nonidet P-40 (NP-40). Subsequent gel filtration determined the molecular weight to be about 35,000 daltons. Isoelectric focusing gave two peaks at pH 4.5-5.0 and pH 5.4. Organic solvent extractions of the thymocytes in acetone:chloroform and acetone only resulted in an 80 per cent loss of activity, while extraction in acetone:water left most of the activity. In all three extractions, the residual activity was associated with the extracted proteins, but could be linearly increased by addition of the extracted lipids or NP-40. These investigators concluded that Thy-1 was a protein requiring nonspecific lipid interaction for Thy-1 activity (29).

<u>Glycoprotein nature of Thy-1</u>. A number of investigators who have studied the Thy-1 antigen of murine system and its analogue in the rat system have concluded that Thy-1 is a low molecular weight glycoprotein (9,27,28,35-37,41).

Trowbridge et al. (35), studying several mouse T lymphoma cell lines, isolated a molecule of 25,000 to 30,000 m.w. which they reported to be identical to Thy-1.1. The cells were lactoperoxidase radioiodinated, detergent solubilized, treated with crude rabbit anti-thymocyte serum (ATS) or ATS absorbed to be made specific for T cell antigens. Biosynthetic labeling studies, sensitivity to proteolytic enzymes, and affinity for plant lectins suggested that this molecule was glycoprotein. No radioactivity was precipitated from NP-40 lysates of cells labeled with ^{3}H-palmitate using a variety of antisera, suggesting that Thy-1 was not associated with lipids. The Thy-1-active antigen was precipitated by rabbit antisera against mouse brain, rat brain, and rat thymocytes, and was not detected on the surface of Thy-1 negative variants of mouse T lymphoma cell lines. Rabbit antiserum to "purified" rat brain Thy-1.1 demonstrated the ability to recognize and bind their "purified" molecule. Mouse anti-Thy-1 antiserum failed to precipitate any ^{125}I-labeled cell surface moiety on the T lymphomas studied, though this was attributed to low affinity antibodies in these antisera or disruption of "multivalent cell membrane interactions" by the presence of the detergent (35).

Another T lymphoma cell line, S-49.1-TB.2.3 (Thy-1.2), was submitted to a limited digestion by papain and the solubilized material partially purified by passage through Sephadex G-200 (37). A single peak was eluted which contained all the inhibitory activity of a ^{51}Cr-release cytotoxicity inhibition system using ^{51}Cr-labeled S-49.1 cells and AKR anti-C3H Thy-1.2 antiserum. Trypsin digestion diminished the inhibitory activity as did treatment with neuraminidase. Since this enzyme cleaves sialic acid residues, the proposal was set forth that

Thy-1 was glycoprotein in nature. Furthermore, sialic acid alone was found to inhibit the anti-Thy-1.2 cytotoxicity for S-49.1 cells, prompting the suggestion that sialic acid was part of the antigenic determinant of the Thy-1.2 alloantigen (37).

Zwerner and his associates (41) also used a T lymphoma cell line in their studies on large scale production of Thy-1 for detailed structural and functional analysis. They used the cell line BW 5147, a spontaneously derived AKR T lymphoma, expressing large amounts of the Thy-1.1 alloantigen. The cells were mechanically disrupted, acetone precipitated, subjected to gel filtration and SDS-PAGE. The "purified" Thy-1.1 was evaluated by a thymocytotoxicity inhibition assay. Based on this study they reported that Thy-1.1 was a glycoprotein of about 25,000 m.w. (41).

In accord with previous findings, McClain (9) isolated and studied the composition of Thy-1.1 and Thy-1.2 in mouse brain tissue, reporting that about 20 per cent of the molecule was carbohydrate. Amino acid and carbohydrate composition data revealed the alloantigens to be almost identical, suggesting the antigenic distinction between Thy-1.1 and Thy-1.2 to be too subtle to be detected by compositional studies alone (9).

Finally, Barclay et al.(27,28,36), isolated and characterized Thy-1 from rat brain, rat thymocytes (27,28), and mouse brain (36). Their method employed solubilization of crude thymocyte and brain cell membranes in deoxycholate, gel filtration on an upward flowing Sephadex G-200 column, and affinity chromatography on a lentil lectin column, or affinity chromatography of the deoxycholate extract of thymocyte membranes on an anti-brain-associated Thy-1 antibody column. A final purification was performed by SDS-PAGE. Rat brain Thy-1.1 was determined to be a glycoprotein of 24,000 m.w. Rat thymocyte Thy-1.1 was isolated in two forms: a moiety of 25,000 m.w. that bound readily to the lentil lectin affinity column, and a molecule of 27,000 m.w. that passed through the column. Amino acid analysis indicated a high degree of similarity between the three forms of Thy-1.1. All three molecules contained approximately 30 per cent carbohydrate, although, in disagreement with McClain's findings (9), analysis of the carbohydrate composition showed great dissimilarities in relative amounts of various sugars (28). The Thy-1 activity of these "purified" molecules, as assayed by a radioactive binding inhibition assay, was destroyed by treatment with pronase. Therefore, they proposed the antigenicity of the Thy-1.1 alloantigen to be found in the protein portion of the molecule (27,28). This evidence is inconclusive since pronase is usually contaminated with other enzymes.

Employing the same techniques, the same group (36) examined mouse brain Thy-1 antigens. Antigenic activity was associated with a glycoprotein of 25,000 daltons, estimated by SDS-PAGE. No carbohydrate heterogeneity was detected in mouse Thy-1 by the separation methods employed. The amino acid composition was similar to that of the rat brain (28). Antigenic studies indicated that heteroantisera raised against the mouse brain Thy-1 glycoprotein was strongly crossreactive with the rat brain Thy-1 moiety and did not discriminate between Thy-1.1 and Thy-1.2 alloantigens. As with some other studies, the presence of detergents inactivated Thy-1, destroying its ability to block the cytotoxicity of specific anti-Thy-1.2 antisera. Removal of the detergent only partially restored activity. This finding was interpreted to mean that anti-Thy-1 antibodies were probably of low affinity, requiring "multivalent antigen for effective displacement of binding to a target cell where the antigen density is high" (36). It also allows for the possibility that Thy-1 activity is associated with a lipid or glycolipid.

Glycolipid nature of Thy-1. A number of researchers, in their attempts to characterize Thy-1, have reported that Thy-1 is sensitive to exposure to lipid solvents and detergents (4,29,31,35,36,38). Reif and Allen (4) described a membrane bound antigen with Thy-1 activity that was sensitive to organic solvent extraction. Kucich et al. (38) submitted their Thy-1 preparations to a Folch partition and found that the "Thy-1 protein" had lost its Thy-1 properties. Arndt and his co-workers (29) also reported a loss of Thy-1 activity from their preparations upon chloroform:acetone extraction, but activity could be restored by adding back the extracted lipids or a small amount of the nonionic detergent NP-40.

In contrast to results reported by Arndt et al. (29), Trowbridge et al. (35) were unable to specifically precipitate any Thy-1 from lysates of NP-40 or deoxycholate-treated thymocytes or T lymphoma cells. Letarte and Meghji (36) encountered a similar problem where Thy-1, isolated in the presence of deoxycholate, was not inhibitory in an anti-Thy-1 cytotoxicity assay. Both groups attributed this phenomenon to low affinity antibodies that required multivalent antigen for an effective precipitating complex to form (35,36). Even so, removal of the detergent restored only a small amount of the original activity (36). None of these groups tested for Thy-1 activity in the lipids removed by their procedures.

Vitteta et al. (31) also had difficulty with the precipitation of Thy-1 in NP-40 lysates of murine thymocytes and T cells. The cell surfaces were radioiodinated and, while specific antisera were capable of precipitating H-2 and TL antigens, Thy-1 activity could not be removed from the NP-40 lysate by congenic anti-Thy-1 alloantiserum. If the lysates were prepared by freeze-thawing, labeled Thy-1 reacted with the specific antibodies. It was proposed that the majority of the radioactivity was present in a molecule noncovalently bound to the antigenic portion of the Thy-1 complex and/or the antigenicity of the complex depended on a lipid moiety dissolved by the detergent. A Folch partition of the NP-40 soluble material resulted in greater than 50 per cent recovery of the radioactivity in the lower (chloroform) phase where labeled lipids and lipoproteins were found. Sedimentation studies showed that Thy-1 sedimented at a lower density than protein, suggesting lipid association with the Thy-1 complex. These researchers also found that they were able to label Thy-1 with ^{3}H-galactose, but not ^{3}H-leucine or ^{3}H-tyrosine. Polyacrylamide gel electrophoresis of the ^{3}H-galactose-labeled moiety gave a broad spectrum of radioactivity peaking at 35,000 m.w., suggesting protein and glycolipid participation in the Thy-1 complex. It was proposed that Thy-1 might be glycolipid in nature, or that the antigenicity of Thy-1 was associated with a glycolipid (31).

In accord with this finding, Rabinowitz et al. (33) reported that boiling of the mouse thymus destroyed the antigenicity of TL and H-2 antigens but not of Thy-1, an expected result if Thy-1 were a glycolipid or if its antigenicity were carried by the carbohydrate portion of the molecule.

More evidence for the glycolipid character of Thy-1 was provided by Miller and Esselman (30,40,43,44). They reported that G_{M1} and G_{D1b} gangliosides were capable of inhibiting the cytotoxicity of specific anti-Thy-1 antisera and anti-brain-associated-Thy-1 serum (30,40). These gangliosides were found to have Thy-1 activity. Further support that the antigenicity of Thy-1 was carried in the carbohydrate portion of the molecule was the finding that pentasaccharides derived from the gangliosides were able to specifically inhibit anti-Thy-1 thymocytotoxicity (45).

G_{M1} ganglioside is known to function as a cell surface receptor for cholera toxin (46). Thiele et al. (47) reported experiments in which fluorescent-labeled cholera toxin (CT) and anti-Thy-1 antibodies induced cocapping on thymocyte cell surfaces. Since pretreatment of the cells with CT did not inhibit anti-Thy-1-dependent cytotoxicity, it was proposed that the CT receptor and the Thy-1 antigenic determinant were on the same molecule but represented spacially separated sites (47). Contradicting results have since been published by DeCicco and Greaves (48) claiming that cocapping cannot be induced by treating murine thymocytes with anti-Thy-1 and CT carrying fluorescent probes. Furthermore, "pure" anti-G_{M1} antibodies have been shown to react equally well with murine thymocytes of either Thy-1 allotype and any H-2 specificity (49) as well as with spleen and lymph node cells bearing Ig surface markers (32).

Recent evidence from the same laboratory that implicated the biological identity of Thy-1 and certain gangliosides (30) has emerged which seems to clear up this controversy. Their further studies suggest that the Thy-1-active glycolipid is present in very small quantities in G_{M1} and G_{D1b} in the previous experiments using these ganglioside preparations (42). In earlier experiments, brain tissue or thymocytes were mechanically disrupted, submitted to a Folch partition, followed by a mild base hydrolysis of the ganglioside-rich upper phase, dialysis, and lyophilization. This material was applied to an Anasil S column utilizing a series of solvent systems of chloroform:methanol:water mixtures. Fractions were collected and submitted to thin layer chromatography (TLC) in a chloroform:methanol:ammonia system (30). Isolation of thymocyte glycolipids omitted the column chromatography separation step. Further fractionation in another TLC system of chloroform:methanol:water resulted in the isolation of a minor component from G_{M1} which contained all the Thy-1 activity. This activity was assessed in an antigenicity assay by its ability to induce an anti-Thy-1 response in allogeneic spleen cell cultures (42).

Disagreement still remains as to the glycolipid or glycoprotein nature of the Thy-1 alloantigen. The variety of approaches to solving this problem has often increased the confusion and led to the reporting of results in conflict with those already in the literature. Whether the antigenicity lies in a protein or lipid, or a carbohydrate moiety that can be shared by either, as with the blood group antigens (50), is an area open to further investigation. Recent evidence, however, suggests that the antigenicity resides in the carbohydrate structure of Thy-1. Wang et al. (42) demonstrated an anti-Thy-1 response when either Thy-1 glycolipid or Thy-1 glycoprotein was incubated with spleen cells of the opposite allotype. The reacting spleen cells were assayed for anti-Thy-1 plaque-forming cells on a lawn of thymocytes exhibiting the Thy-1 allotype of the isolated inducing antigen.

Biological Role of Thy-1

Introduction. The discovery of a biomolecular complex unique to a system raises several interesting questions and warrants intensive research to discover their answers. The uniqueness of a molecule to a system or a cell type, its ontogeny, its biophysical properties, its location on or within a cell, represent information which must be explored for important clues to the teleology of the complex. To this point in time, the majority of those investigations of the Thy-1 alloantigen have concentrated on documenting its presence, nature, and its uniqueness as a T lymphocyte marker. It has been identified in mouse (1,4), rat (22), and possibly human tissue (25). Although it can be serologically identified in other tissues, it has been found to be a convenient T cell membrane marker in the mouse (16), and its appearance during T lymphocyte maturation has been studied (8,19,20,51). A variety of products elaborated from lymphocytes carrying this marker is reviewed in a later section of this paper. Few investigators, however, have confronted the problem of what biological role Thy-1 may play, if it has a function beyond that of its involvement in T lymphocyte differentiation and maturation.

B cell modulation by shed Thy-1 antigen. An interesting model of immunugenesis has been proposed in which a T cell factor interacts with the lipid membrane of the B lymphocyte, directing its differentiation toward eventual antibody production (52). This is also considered a possible mechanism of interaction of LPS with B cells, in which the lipid A portion of the molecule interacts lipophilically with the B cell membrane. Miller and Esselman (43,44) have proposed a similar mechanism for the interaction of a T cell product, a glycolipid with Thy-1 activity (30), with B lymphocytes. This model begins with antigen-stimulated T cells releasing membrane fragments containing Thy-1 which then interact nonspecifically with surrounding B cells and protectively block direct antigen binding to antigen-specific B cells, a condition that would result in tolerance and/or unresponsiveness. The proposed regulation is temporary until T helper factors can accumulate and become involved in the response (43). Indeed, studies have shown that injections of near-syngeneic thymocytes are able to prevent the induction of B cell tolerance to a T-dependent antigen in nu/nu (congenitally athymic) mice (53). Similar results were reported in _in vitro_ studies (54). It was discovered that prior addition of polimerized flagella (POL) could protect B cell cultures from tolerance induction due to large doses of a T-dependent antigen (54). POL may have provided steric hindrance disallowing direct antigen binding to B cells, thus protecting them from tolerance.

Miller and Esselman (40) provided additional support for their model when they extracted glycolipids that exhibited Thy-1 activity from mouse brain. These glycolipids which comigrated with G_{M1} and G_{D1b} gangliosides on TLC (using earlier technology), were then prepared into liposomes and incubated with spleen cell cultures for five days with sheep erythrocytes (SRBC) as the test antigen. Subsequent hemolytic plaque-forming cell (PFC) responses to SRBC were evaluated and substantial suppression of the anti-SRBC PFC response in the treated cultures was noted when compared to the controls. The effect was most dramatic when Thy-1 glycolipid was added on the first day of the culture, although some suppression was seen if it was added on days 2 and 3. Further investigations were conducted (43) in which mice were immunized with a high dose of antigen (10^9 SRBC), their spleens removed two days later, enriched for T cells by passage over glass and nylon wool columns, and cultured for three days. When added to bone marrow-thymocyte cultures, the 3-day culture medium was able to suppress anti-SRBC PFC responses. Nonspecificity of this phenomenon was demonstrated in normal spleen cell cultures stimulated by burro red blood cells (BRBC) or trinitrophenylated-SRBC (TNP-SRBC). Addition of the suppressor medium from the T lymphocytes, originally stimulated _in vivo_ by SRBC, abrogated the PFC response to both heterologous antigens. Extraction of the glycolipids from the medium resulted in the isolation of a glycolipid with G_{M1} mobility on TLC that had nonspecific suppressive properties in a PFC response assay. The bone marrow-thymocyte cultures were maximally suppressed by day 12 for the IgM response, and day 11 for the IgG response. When PFC responses were measured 48 hours later, they had increased to peak levels beyond the peak responses of the control cultures. Hence, the suppression was only temporary and was followed by an increased response in the Thy-1-treated cultures (43). The suppressor activity in the supernatants of the SRBC-primed T cell cultures could be absorbed out with anti-Thy-1 and, to a lesser extent, with anti-G_{M1} antisera, allowing the PFC response to return to the level of the controls (43).

Corrêa et. al. (55) have studied a system of antigenic competition in which Thy-1 glycolipid appears to play a key role. Spleen cell cultures from TNP-bovine gamma globulin (TNP-BGG)-primed mice were immunized with ovalbumin (OVA) and then given a second antigen, TNP-BGG, 24 hours later. This resulted in a weak anti-TNP hemolytic plaque response compared to controls that did not receive OVA. This apparent state of antigenic competition was overcome by absorbing the culture medium with anti-Thy-1 or anti-G_{M1} antisera. The culture media of various treated and control groups were submitted to a lipid extraction, and a glycolipid with Thy-1 activity was found to be responsible for the suppressive activity (55). Further investigation showed that if B cell cultures were allowed to react directly with large amounts of antigen (SRBC), followed by addition of T cells, they were unresponsive in an anti-SRBC PFC assay. However, if the cultured B cells were treated with Thy-1 glycolipid first, followed by antigen and then T cells, or the T cells were allowed to react with the antigen before antigen and T cells were added to the B cell cultures, a near normal PFC response ensued. The results presented by these workers provided support for the proposal that antigen-stimulated T cells release Thy-1 as a modulatory signal, preparing B cells for further stimuli leading to terminal differentiation (55).

The ultimate test of an _in vitro_ phenomenon is its applicability to an _in vivo_ system. While the above studies on the functional role of Thy-1 are instructive, it has yet to be established whether this is an _in vitro_ artifact or, indeed, an _in vivo_ reality.

Thy-1 shedding from T lymphocytes. Shedding, the phenomenon of selective release of membrane constituents into the extracellular environment from viable cells (56), is seen as an important process in release of Thy-1 antigen from lymphocytes (43,44,57,58). Whether membrane elimination is a normal maintenance process in the turnover of membrane constituents, or serves a more important role is not fully understood. Little is known about the mechanism of shedding. It has been suggested that surface proteases are involved or that clasmatosis, the pinching off of microvilli, is important in the process (56). The temperature dependence of plasma membrane shedding implies enzymatic or metabolic control (56).

Release of membrane-bound constitutents has been described in a number of malignant (39,59-63,69) and nonmalignant cell types (43,44,58, 64-68). Shedding of surface complexes has been documented in human (59) and murine (63) cells. Several mouse T lymphoma cell lines (39) and at least one human T lymphoma (69) are known to rapidly shed their surface antigens. Correlations between rates of shedding, rates of tumor growth, immune response suppression, and tumor metastises have led to the suggestion that shedding is an important mechanism for tumor escape from immune destruction (60-62).

Shedding has been described in some nonmalignant cells and appears to be a normal process under certain conditions. Embryonic and fetal cells shed cell surface antigens rapidly, perhaps to avoid immune destruction by maternal antibodies before they can bind to embryonic tissue (61). Lutz et al. (64) reported that under conditions of ATP depletion, aged human red blood cells (HRBC) shed surface complexes in the form of small liposomes. Selectivity of the process was evidenced by the fact that normal amounts of all membrane proteins were present in the liposomes except spectrin, a major HRBC membrane-bound protein, which was absent (64).

Considerable attention has been given to a variety of biomolecules elaborated from various lymphoid cell types. Vitteta and Uhr (68) used lactoperoxidase radioiodination of cell surface antigens to observe the release of cell surface Ig noncovalently bound to a fragment of plasma membrane. Shedding was selective as H-2 antigens were not shed during the short term incubation used to detect the shed Ig. Cone et al. (65), used the same labeling technique with thymocytes, bone marrow cells, and thoracic duct cells to study lymphocyte shedding. Use of metabolic inhibitors indicated that cellular respiration and protein synthesis were indispensible for this process (65). In yet another study, radioiodinated T cells were shown to release 90% of the surface label in the first hour of incubation in culture, while maintaining 85% viability (67). The radioactive shed complexes could be absorbed by Phytohemagglutinin (PHA) or Concanavalin A (Con A) treatment of the medium. Treatment of the cells with PHA or Con A increased the rate of shedding. Absorption of the media with anti-PHA or anti-Con A antisera resulted in the precipitation of complexes consisting of PHA or Con A and their respective shed membrane receptors (67).

Differential shedding from T lymphocytes was described in radiolabeling studies conducted by Vitteta et. al. (58). By labeling murine thymocytes with ^{125}I, ^{3}H-galactose, or ^{3}H-leucine, they were able to show preferential shedding of Thy-1 antigen from T cells while H-2 antigen was either not shed or was eliminated at a much lower rate. These authors proposed Thy-1 to be a peripheral membrane moiety, while H-2 was probably a more integral membrane constituent (58). Lake (57) reported the induction of a primary anti-Thy-1 response _in vitro_ by medium from a murine T cell culture of one allotype when it was introduced to a spleen cell culture of the other allotype, as assayed by an anti-thymocyte

plaque technique. This was interpreted as evidence that Thy-1 antigen was shed by thymocytes in culture (57).

Finally, Miller and Esselman (43,44)) reported that medium from a T cell culture, prepared from mice primed in vivo with a high dose of antigen (10^9 SRBC), contained Thy-1 activity in glycolipid form. Furthermore, in antigenic competition studies, medium from OVA-stimulated cultures of spleen cells previously primed in vivo with TNP-BGG, exhibited the same activity which could be attributed to the presence of the same glycolipid moiety, suggesting the selective release of Thy-1 from T cells during antigen stimulation (55).

The nature of the shed complexes is still an area for continuing research. Electron microscope studies of Lutz et al. (64) on shedding from ATP-depleted human red blood cells indicate that membrane fragments were released as small vesicles of liposomes of uniform size and shape. Surface antigen shedding in liposome form has also been observed in mouse mammary tumor virus (MuMTV) studies of GR Ascites leukemia cells (61). Release of the Thy-1 and H-2-containing vesicles could be stimulated by treatment of the cells with rabbit anti-MuMTV antiserum (61).

In their investigation of Thy-1 dependent modulation of B cell differentiation, Miller and Esselman (40,43,44,55) found it necessary to incorporate Thy-1 glycolipid into liposomes made up of auxillary lipids, cholesterol and lecithin (C:L). When Thy-1 glycolipid or C:L liposomes were used alone in their culture systems, no significant effect on B cells was seen. However, when Thy-1 was incorporated into the C:L liposomes, B cell modulation again became evident (40). They proposed a model in which Thy-1 was shed from activated T cells in complex with other membrane lipids, forming liposomes. The liposome form appeared to be necessary for Thy-1 glycolipid activity (43,44).

Role of T Lymphocytes in Humoral Immune Response Regulation

Lymphocyte Interactions

It has been demonstrated in recent years that regulation of the humoral immune response, both *in vitro* and *in vivo*, requires interaction between thymus-derived lymphocytes (T lymphocytes) and precursors of antibody-producing cells, B lymphocytes (70-74). By reconstitution of irradiated or irradiated/thymectomized mice with combinations of thymocytes, bone marrow cells, and thoracic duct cells, Claman et al. (70) and Miller and Mitchell (71,73) demonstrated T cell - B cell synergism for host antibody responses to most antigens. T cells appeared to be regulatory while B cells eventually became the antibody-producing cells. The first regulatory function attributed to T cells was that of a helper function as exhibited in the above studies. In contrast, Gershon and Kondo (75) observed that specific immunological unresponsiveness in mice given high doses of sheep erythrocytes could be passively transferred to normal mice by the transfer of T cells from the tolerant mice to the unimmunized mice. This observation established that, under suitable conditions, antigens could induce the differentiation of a subpopulation of T lymphocytes capable of suppressing the formation of antibody-producing cells (75). Thus, T cells could be categorized as to their ability to exert a positive (helper) or negative (suppressor) influence on the humoral immune response. Development and function of T lymphocyte subpopulations have been reviewed in detail (76,77).

Mechanisms of T Cell Regulation of the Humoral Immune Response

Extensive work has been done in an effort to elucidate the role of the T cell subpopulations in control of the immune response, although the precise mechanisms of this system of "fine tune" regulation remain to be defined.

Cell-to-cell contact. Several investigators have shown that cell-to-cell contact or close association is necessary for the induction, proliferation, and differentiation of B cells into mature antibody producing cells (78-80). Working independently, Mosier (78) and Pierce and Benacerraf (79) produced evidence of antigen-induced lymphocyte-macrophage clustering *in vitro*. This interaction was specific (80), mediated by antigen and/or immunoglobulin (Ig) on the surface of the interacting cells, and was required at least during the early stages of the primary response (78,79). The cells could then be dispersed and activated cells could continue to develop into antibody-forming cells, independent of other cells and clusters (79). McIntyre et al. (80) produced ultrastructural studies confirming septate-like junctional complexes between lymphoid cells antigenically stimulated in culture, although no group distinction between the lymphoid populations was possible.

Soluble mediators of regulation. Considerable evidence points to the importance of soluble mediators of immunoregulation produced and released by macrophages (77,81) and T lymphocytes (43,44,69,76,84-90,92,94-101) in directing the proliferation and differentiation of B cells without cell-to-cell direct contact. The *in vivo* induction of antibody-forming cells probably requires a combination of specific cell-to-cell contacts among macrophages and lymphocytes and elaboration of soluble mediators effective over a short distance in the microenvironment surrounding the cell types active in the response.

Soluble T helper factors. A number of researchers have isolated and studied cell-free helper substances released from antigen-stimulated spleen cell cultures (82,83). These factors have been shown to replace T cells both the *in vivo* and *in vitro* induction of the immune response. Rubin and Coons (84) first described a factor isolated from the supernatant of spleen cell cultures or mice primed 30 to 60 days before culture with tetnus toxin (TT). Addition of nanogram amounts of TT to cultures being stimulated with sheep red blood cells (SRBC), enhanced the anti-SRBC response significantly over the control cultures. The responsible factor was nondialyzable, stable for 30 minutes at 56 C, and inactivated by proteases but not DNase or RNase (84). Gorczynski et al. (85) and Watson (86), using slightly different methods, also described the presence of a similar factor in antigen-stimulated cultures of spleen cells from irradiated mice (86). This factor was proposed to be released from specifically activated T cells, although nonspecific in its ability to enhance the humoral response to an unrelated antigen (84-86).

Taussig (87) described a factor which was present in the medium of cultures made from "T cell spleens" from irradiated, thymocyte-reconstituted mice, subsequently immunized with a T-dependent antigen. The medium containing the cell-free factor was mixed with bone marrow cells and antigen and transferred to lethally irradiated recipients. The humoral response was measured at a later time and found to be equal to that of the control mice which received either the "educated" T cells (from mice already exposed to the antigen) or normal T cells. This helper substance was found to be antigen-specific in its effect, as transfer of the supernatant combined with bone marrow cells and an antigen unrelated to the primary antigen failed to elevate the response to the unrelated antigen compared to the controls (87).

Armerding et al. (88) have studied a factor elaborated by alloantigen-activated T cells in a mixed lymphocyte culture. Short term incubation of T cells, previously primed with histoincompatible lymphocytes *in vivo*, with the appropriate target cells produced an "allogenic effect factor" (AEF), elaborated into the culture medium capable of nonspecifically restoring the anti-SRBC and anti-dinitrophenol responses in T cell-depleted spleen cell cultures (88). This was attributed to its ability to replace the need for carrier-specific helper T cells. AEF was further characterized as being a glycoprotein of two subunits, 40,000 and 12,000 daltons, both necessary for activity (89). Production of this factor was dependent on H-2 differences of the cells in the culture (90). DNA replication and proliferation were not necessary, while glycolysis, protein synthesis, and electron transfer were prerequisites to the synthesis and release of AEF (90). Phenotypic studies on T cell subpopulations indicated that T cells exhibiting Ly-1 and Ly-2,3 cell surface antigens were required for AEF production (90). By using specific anti-Ly antisera, Jandinski et al. (91) confirmed Ly-1 positive T cells as those responsible for helper activity and identified T cells exhibiting the Ly-2,3 phenotype as those responsible for suppressor functions.

Soluble T suppressor factors. A multitude of soluble T cell-generated immune response suppressor substances have been described. Suppressor T cells and their products have been characterized as antigen-specific (76,92,93) or antigen-nonspecific (43,44,69,86,94,96-101) in their inhibitory activities. Suppression of the immune response by soluble T cell factors may provide at least partial explanations for antigenic competition (55), tolerance (75), mixed lymphocyte reaction (101), and modulation of the humoral immune response (43,44,69,76,86, 94-101).

The apparent diversity of factors in their biological as well as their physiochemical properties can probably be attributed to the variety of methods used by investigators to isolate and study them. Takemori and Tada (92) extracted a factor from physically disrupted thymocytes and spleen cells of mice formerly immunized with the soluble hapten carrier, keyhole limpet hemocyanin (KLH). Injection of this factor into syngenic mice immunized with dinitrophenol-KLH (DNP-KLH) significantly suppressed the secondary (IgG) anti-DNP response (92) as measured by a hemolytic plaque technique. The activity could be removed by absorption with heterologous anti-thymocyte serum, alloantibodies against the donor strain, and antigen, but not by anti-mouse immunoglobulin antibodies. This antigen-specific, heat labile protein of 35-60,000 daltons was suppressive in mice with same H-2 specificity and had no effect in allogeneic mice (92). Further studies showed that this substance exerted its inhibitory influence on T helper cells rather than B cells, providing evidence for interactions among T cell subpopulations as well as T cell-B cell communication (76).

A factor similar in many respects to that described by Takemori and Tada (92), was investigated by Kapp et al. (93) using mice classified as nonresponders to the synthetic polypeptide L-glutamic acid^{60} - L-alanine^{30} - L-lysine^{10} (GAT). Priming with GAT specifically decreased the ability of these mice to respond to GAT-methylated bovine serum albumin (GAT-MBSA) or GAT-pigeon red blood cells (GAT-PRBC), two forms of the antigen that were normally immunogenic (93). T cells from these mice were extracted according to the procedure of Takemori and Tada (92). The T cell extract was then introduced into normal syngeneic mice together with GAT-MBSA or GAT-PRBC. Again the response was suppressed. The antigen specific factor in the T cell extract could be absorbed by GAT-Sepharose (but not BSA-Sepharose), was removed by alloantisera to the Ir gene products, and eluted from a Sephadex G-100 column with a molecular weight of 45,000 daltons (93).

Following a protocol similar to that described for his helper factor (87), Taussig (94) used the spleens from irradiated thymocyte-reconstituted mice which were subsequently primed with a T-dependent antigen *in vivo* and removed 7 days later for culture. The cultures were exposed to the primary antigen and found to develop suppressive function not observed in previous cultures not exposed to the immunizing antigen *in vitro* (94). He attributed the suppressive activity to the possible presence of a soluble suppressor factor elaborated by a subpopulation of T cells, distinct from the helper T cell population, which outlasts the helper factor. That is, the suppressor factor was more stable or it eventually overrode the helper factor effect (94).

Others have elicited production of nonspecific suppressor factors, employing a host of similar methods of antigen or mitogen activation of T cells. Most methods involved priming the experimental mice with antigen several days before collecting the spleens for culture (43,93-97). In some studies, the spleen cells required incubation with additional antigen in culture (94,96). In one report, a single exposure to antigen *in vivo* produced a helper effect, and a second exposure *in vitro* elicited the suppressor activity (Taussig - compare 87 and 94). Another required "double priming" with two antigens for the suppressive factor release; priming with a single antigen gave an enhancing factor (96). Yet another showed that priming *in vivo* with a low dose of antigen (10^6SRBC) influenced the release of helper activity from T cells in culture, while prior exposure to a high dose (10^9 SRBC) resulted in a suppressive factor being released into the medium from the cultured T cells (43). Many of the substances investigated fell in the molecular weight range of 35-60,000 daltons (93,95,97,98), with some exceptions where the molecular weights were considerably less, i.e., less than 10,000 daltons (43,99). When characterized, most of these were found to be glycoprotein (95,97) but no further properties were available. In one case (43), the molecule responsible for suppression was defined as a glycolipid with the mobility of G_{M1} ganglioside by thin layer chromatography and was associated with activity of the T cell surface antigen, Thy-1 (43).

Con A has been used for multiclonal activation of spleen cells in culture to elicit suppressor factor release into the medium (98,101). Rich and Pierce (100) isolated and characterized a "soluble immune response suppressor" (SIRS) and showed its ability to suppress the primary response in spleen cell cultures to heterologous erythrocytes without affecting viability (100). SIRS presence was only required for the initial 24 hours of the response and the suppression was evident by day 5 of the culture. SIRS was determined to be a glycoprotein of 48-67,000 m.w. It was not reactive with anti-IgG or anti-μ chain, could not be absorbed by antigen, and lacked I region determinants (98). In addition, SIRS could not be separated from migration inhibition factor (MIF) activity (98). Further studies determined that SIRS was elaborated from a T cell subpopulation bearing the Ly-2,3 phenotype (91). Reinertsen and Steinberg (99) isolated a molecule from Con A-stimulated spleen cell culture supernatants which contained suppressor activity, was less than 10,000 m.w., and lacked MIF activity. This suggests separation of the active part of the SIRS molecule from that with MIF activity.

Work has been done on a human T lymphoid cell line, MOLT-4 (69), which forms SRBC rosettes, has no surface immunoglobulins, no complement receptors, and no receptors for antibody-antigen complexes. Anti-MOLT cytotoxicity could be absorbed with fresh human thymocytes. When exposed to SRBC, but not chicken erythrocytes (CRBC), MOLT elaborated nondialyzable immunosuppressive factors into the medium which were capable of suppressing the anti-SRBC and the anti-CRBC responses in mouse spleen cell cultures (69).

In vivo applications. The ultimate test of any helper or suppressor factor is demonstration of its role in vivo. As fulfillment of this criterion, Taussig (94) reported replacement of T cells with a T cell factor in an in vivo response. Irradiated, thymocyte-reconstituted mice were primed with the highly T-dependent synthetic polypeptide poly (Tyr-Glu)-poly DL Ala--poly-Lys, abbreviated (T,G)-A--L. Their spleens were subsequently removed and cultured. The culture supernatant, bone marrow cells, and (T,G)-A--L were injected into lethally irradiated syngeneic recipients. Twelve days later, spleens were removed and subjected to a direct (IgM) hemolytic plaque-forming-cell (PFC) assay for evaluation of antibody-forming cells. A significant increase in PFC over controls was observed. Induction of the helper activity was antigen-specific while its effect was antigen-nonspecific (87).

Takemori and Tada (92) reported in vivo suppression of the indirect (IgG) anti-DNP response, using a factor extracted from carrier-primed T cells. This antigen-specific factor was coded for by the I-J region of the H-2 complex (102) and was active only in H-2 histocompatible hosts (92).

A further demonstration of in vivo biological activity was provided by Kapp and her associates (93) in experiments with a factor extracted from antigen-primed T cells. This factor, extracted from GAT-primed T cells from nonresponder mice, was injected into normal syngeneic mice, suppressing the immune response to GAT-MBSA and GAT-PRBC, antigens which would normally elicit a response.

In an interesting set of experiments, Krakauer et al. (102) drew a connection between SIRS production (100) and age-dependent development of an autoimmune syndrome by NZB/NZW mice. They attempted to elicit SIRS production by Con A stimulation of T cells from these mice at various ages and measured its effect on a defined immune response.

A strong correlation between declining SIRS production, increase in age, and increase in development of autoimmunity was reported (102). These authors proposed that this was due to fewer SIRS-producing cells with the increase in age and the animal's incapacity to suppress "forbidden clones" acting against "self" tissue antigens (103).

Until this battery of helper and suppressor substances is purified and biochemically characterized, it is difficult to do more than speculate about the mechanisms of action on the molecular level. More understanding is required about the nature of the mediators, their active site(s), their interactions with cell membranes, their effects on cyclic nucleotides, intracellular divalent cations, microtubules and microfilaments (83). Regulation of the immune response appears to be a complex interrelated network of finely tuned systems of which soluble mediators are only one important part. Useful manipulation of the immune system will require a much greater understanding of all facets of the network.

In summary, the discovery of the dichotomy of the cell types participating in the humoral immune response has stimulated a great deal of research directed toward elucidating the nature of the cellular interactions which occur. Efforts have been aimed at methods of identifying the specific cell types involved and the mechanisms of their cooperation. The Thy-1 alloantigen, a convenient cell surface marker for identifying T lymphocytes in the mouse, has been helpful in studying the subpopulations of lymphocytes which appear to play a supervisory role in controlling and fine tuning the humoral immune response. Controversy still exists as to its biochemical nature, though it may be that it exists in both a glycolipid and glycoprotein form with its antigenicity residing in the carbohydrate portion. *In vitro* studies suggest B cell modulation as the biological role of Thy-1. An *in vivo* functional role for Thy-1 has yet to be established.

MATERIALS AND METHODS

Mice

C3H x C57BL/10 Cz (Health Research, Inc., Buffalo NY) and C3H x C57Bl/6 He (Cumberland View Farms, TN) female mice were used to evaluate antibody responses following imposed treatments. Mice of both substrains were referred to as BC3F$_1$ by their respective suppliers. For clarity, they will be referred to as B10C3F$_1$ and B6C3F$_1$ in this report. Mice were 8 to 15 weeks old and were age-matched for a given experiment.

Sheep Erythrocytes

Sheep erythrocytes (SRBC) to be used as antigens were obtained from a single tested sheep (735 Blk) at Grand Island Biological Co., Madison, WI. SRBC were stored in Alsever solution before use and washed three times in sterile phosphate-buffered saline plus 0.2% glucose (PBS plus glucose), the buffy coat removed and discarded, and the cells resuspended in PBS plus glucose for *in vivo* immunization or in Minimal Essential Medium (MEM, Hank's base, Grand Island Biological Co., Grand Island NY, pH 7.2-7.5) for use in the hemolytic plaque assay.

Hemolytic Plaque-Forming Cell Assay

The *in vivo* induction of primary and secondary humoral immune responses in mice was measured by the slide modification of the hemolytic plaque assay as described by Miller and Cudkowicz (104). Mice were primed with 5 x 10^7 or 5 x 10^8 SRBC by intravenous injection in the lateral tail vein. Each individual spleen was removed 3 to 11 days later, minced with forceps in 1.0 ml of MEM, followed by gentle aspiration with a syringe and needles of increasing gauge (21 to 27) to obtain a single cell suspension, and placed on ice for immediate use. The cells were diluted in MEM to permit detection of 50 to 500 PFC/0.1 ml. One tenth ml of the appropriate dilution was combined with melted agarose (53 C, Indubiose, L'Industrie Biologique Francaise, S.A.) and SRBC and plated onto microscope slides previously coated with 0.1% agarose and dried. The slides were inverted on special lucite trays and incubated 1-1½ hours at 8% CO_2 tension in a 37 C humid incubator.

Assessment of IgM-producing cells (direct PFC) was carried out using guinea pig complement (fresh frozen, Colorado Serum Laboratories, Denver, CO, and Suburban Laboratories, Silver Spring, MD) diluted 1:10 or 1:20 in MEM, and added to the trays which were incubated for an additional 2 to 3 hours as described above. A duplicate set of slides was prepared for detection of IgG-producing cells (indirect PFC) to which goat anti-mouse IgG (Meloy, Springfield, VA) was added (diluted 1:100 in MEM) after the initial 1 hour incubation. This antiserum was 70% to 80% inhibitory for direct PFC, so appropriate adjustments were made for indirect PFC counts. The slides exposed to anti-mouse IgG were incubated for 1 hour, the antiserum drained off and replaced with complement, and incubated for an additional 2 to 3 hours before reading indirect PFC.

Both direct and indirect anti-SRBC PFC were determined using duplicate slides and the average taken as the direct or indirect PFC/spleen for a given animal. The PFC/spleen for a given treatment group was calculated from groups containing 3 to 6 mice/group. Treatment groups were of equal size for a given experiment.

Occasional discrepancies from experiment to experiment between PFC readings of control groups receiving comparable treatment were encountered, therefore, data for each experiment was standardized to the SRBC control given the value of 100.0, with the exception of PFC data for Figure 4. PFC response for each mouse of each group was adjusted relative to this value, then group means and standard errors calculated. These values are referred to as "adjusted PFC/spleen." For reference, the actual group mean PFC/spleen for the control group is indicated for each experiment.

Isolation of Glycolipids

Glycolipids were prepared by extraction of AKR, C3H, or CBA mouse brain tissue with choloroform:methanol mixtures (105). Extracts were submitted to a Folch partition (106) and the upper ganglioside-rich phase dialyzed against several changes of distilled water for 24 to 48 hours at 4 C. The remaining material was lyophilized, subjected to mild alkali hydrolysis with 0.6N NaOH in methanol, and dialyzed against several changes of distilled water for 24 to 48 hours at 4 C. The remaining sample was lyophilized and separated by column chromatography on Anasil S (Analabs, Inc., North Haven, CT) with chloroform:methanol: water ($C:M:H_2O$) mixtures. The $C:M:H_2O$ mixtures were prepared in the following proportions:

Solvent	C : M : H_2O (v/v/v)
A	65: 25: 4
B	65: 30: 6
C	60: 35: 8
D	60: 40: 10

This sample was applied to the column and 400 ml of solvent A was allowed to flow through the column as void volume at the rate of 20-27 ml/hour. The next 200 ml were collected in 10 ml fractions. Solvent B was then substituted for solvent A and 500 ml collected in 10 ml fractions. Solvents C and D were substituted in turn and 500 ml of each collected in 7.5 ml fractions. Samples of the fractions collected were chromatographed on Silica Gel G thin layer plates (0.25mm thickness, E Merck, Darmstatt, W. Germany) in a solvent system comprised of chloroform:methanol: 2.5 N NH_4OH, 100:42:6, v/v/v. The glycolipids were then visualized with resorsinal and heat and compared to a standard. Fractions containing G_{M1} ganglioside plus Thy-1-active glycolipid were pooled. This material and other gangliosides were quantitated by the method of Svennerholm (107). Several of the glycolipid preparations were provided Dr. Walter Esselman of the Dept. of Surgery, Michigan State University, East Lansing, MI.

Liposome Preparation

Liposomes were prepared for in vivo use and electron microscopy by combining the isolated glycolipid, cholesterol, and lecithin (cholesterol and lecithin from Supelco, Inc., Bellefonte, PA) in the ratio of 1:5:5 (w/w/w). This mixture was dried and sterile 0.85% saline added to obtain the desired concentrations. The combined lipids were then sonicated at 60 Hz (low frequency - LF liposomes - see Figure 1a) in an ultrasonic cleaner (Mettler Electronics Corp., Anaheim, CA) or at 20,000 Hz (high frequency - HF liposomes - see Fibure 1b), using a probe sonicator (Branson Sonifier Cell Disruptor 200, Branson Sonic Power Co., Danbury, CT) for 10 minutes, under $N_{2(g)}$ in an ice bath.

For comparison in the electron microscope, liposomes were prepared in PBS plus glucose and sonicated at 20,000 Hz as before (Figure 1c). The resultant liposome suspensions were used immediately and/or after 24 hours depending on the experiment. In one case, liposomes sonicated at 20,000 Hz were diluted and resonicated for 1 minute at 60 Hz before use.

Liposomes prepared at low frequency (Figure 1a) were generally larger than high frequency liposomes (Figures 1c and 1d), ranging in diameter from 0.05 μm to 1.65 μm or more, while HF liposomes ranged from 0.02 μm up to 0.30 μm. Although size heterogeneity was observed for both preparations, the majority of the liposomes fell within the ranges indicated. HF liposomes prepared in PBS plus glucose consisted of some unilamellar and many multilamellar vesicles, although more than three bilayers were rare. All liposomes in this preparation were within the expected range for an HF preparation, but appeared to be slightly smaller on the average and more homogeneous in size than those prepared in physiological saline. Both LF and HF liposome preparations displayed occasional tightly formed multilamellar concentric vesicles (Figure 1b), though these were rare.

Multilamellar liposomes were present in both HF and LF preparations. LF liposomes appeared to have more concentric superficial

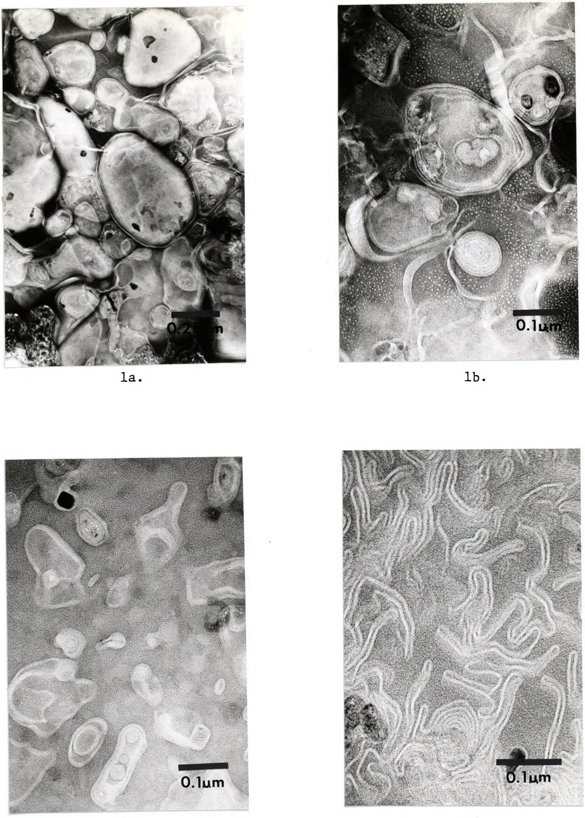
Figure 1. Effect of sonication on liposome morphology. Liposomes were prepared from cholesterol, lecithin, and G_{M1} ganglioside, sonicated, dried, and negatively stained with 2% ammonium molybdate for observation under the electron microscope.

1a. Low frequency (LF) liposomes formulated in 0.85% saline, sonicated at 60 Hz. (Magnification: 48,600 x).

1b. High frequency (HF) liposomes formulated in 0.85% saline, sonicated at 20,000 Hz. (Magnification: 130,500 x).

1c. HF liposomes formulated in PBS plus glucose. (Magnification: 134,500 x).

1d. "Free lipids" (non-liposome lipids, present in LF liposome preparation. (Magnification: 167,500 x).



1a.

1b.

1c.

1d.

Figure 1.

layers and a greater number of inner nonconcentric vesicles than HF liposomes. Length of sonication generally affected the amount of "free" lipids detectable as nonliposome structures (Figure 1d), decreasing somewhat with increased sonication time. Despite the length of sonication, free lipids were always present in LF preparations and to a lesser degree in HF preparations.

Electron Microscopy of Liposomes

Liposomes obtained as described were prepared for electron microscopy by allowing a drop of the suspension to dry on a 300 mesh copper grid coated with a film prepared from 0.5% parlodian (Mallinckrodt Chemical Work, St. Louis, MO) in amyl acetate (108). Each grid was negatively stained (109) with a 2% solution of ammonium molybdate (pH 7.65) for 5 seconds. The specimens were examined and photographed in a Philips 300 microscope.

Statistical Analysis

Comparisons of mean PFC responses of experimental groups of mice were made employing a two-tailed Student's t test.

RESULTS

Attempts to Modulate Antibody Responses In Vivo with Thy-1

It has previously been shown that G_{M1} ganglioside, containing Thy-1-active glycolipid (40), has a modulatory effect on B cell differentiation *in vitro* (42). Initial experiments were performed to test the possibility of an in vivo effect of the Thy-1 glycolipid. B10C3F$_1$ mice (Thy-1.2) were injected i.v. on day 0 with 5 x 10^8 SRBC, followed 24 and 48 hours later by i.v. injections of various glycolipids formulated into cholesterol-lecithin liposomes. Six days after receiving SRBC, spleens were removed and evaluated for the number of anti-SRBC plaque-forming cells (PFC)/spleen. The control group received SRBC followed by PBS injections in place of glycolipids and the response was assigned the arbitrary group mean value of 100.0. All other data were adjusted relative to this value. Results are given in Table 1. CBA G_{M1}(containing Thy-1.2 glycolipid) was found to suppress the anti-SRBC plaque response by about 60% in the direct (IgM) PFC assay compared to the control group in Experiment 1. CBA G_{D1b} ganglioside demonstrated suppression of 38%, while AKR G_{M1} (containing Thy-1.1 glycolipid) was much less effective in suppressing the PFC response, decreasing the response by 24% from control values. The indirect (IgG) response was suppressed by almost 98% in the CBA G_{M1}-treated group and was suppressed to a lesser, but significant, degree with both CBA G_{D1b} and AKR G_{M1} gangliosides.

Another preparation of brain G_{M1} from C3H mice was found to be as effective as the CBA preparation in suppressing the plaque response,

TABLE 1

Suppression of anti-SRBC response <u>in vivo</u> by mouse brain gangliosides incorporated into liposomes

Treatment[a]		Experiment 1 Adjusted anti-SRBC PFC/spleen[b]		Experiment 2 Adj. anti-SRBC PFC/spleen[b]
Day 0	Days 1 and 2	Direct PFC	Indirect PFC	Direct PFC
-	-	0.5 ± 0.2	0.4 ± 0.04	N.D.
SRBC	-	100.0 ± 42.3 (39,900)[c]	96.0 ± 27.0	100.0 ± 9.3 (8,430)[c]
SRBC	CBA G_{M1} (+ Thy-1.2)	40.5 ± 4.2*	2.0 ± 1.0**	18.5 ± 3.8**
SRBC	CBA G_{D1b}	61.5 ± 14.5	9.8 ± 5.3**	N.D.
SRBC	AKR G_{M1} (+ Thy-1.1)	76.5 ± 26.1	18.2 ± 12.7***	N.D.
SRBC	C3H G_{M1} (+ Thy-1.2)	N.D.	N.D.	18.3 ± 1.9**
SRBC	C3H G_{D1a}	N.D.	N.D.	96.3 ± 18.4

(a) Each mouse received 5 x 10^8 SRBC i.v. at 0 hrs. (Day 0) and 2 µg (Exp. 1) or 1 µg (Exp. 2) of the designated glycolipids as indicated incorporated into LF liposomes (see Materials and Methods). The number of PFC/spleen was evaluated 6 days after SRBC injections.

(b) Adjusted mean (see Materials and Methods) ± std. error for 4 mice/group (Exp. 1) and 5 mice/group (Exp. 2).

(c) Actual PFC/spleen.

Student's t-test (compared to SRBC controls) * $P=0.20$ ** $P<0.001$ ***$P<0.01$

while CBA G_{D1a} ganglioside did not deviate from control values (Experiment 2, Table 1). The suppression by G_{M1} treatment was much more dramatic, abrogating more than 80% on the response.

It is notable that the unadjusted direct PFC/spleen values in Experiments 1 and 2 of Table 1 show a great disparity. Experiment 1 produced a direct PFC control response of 39,900 PFC/spleen compared to 8,430 PFC/spleen in the control group of Experiment 2. Control mice in the study shown in Table 2, which received identical treatment as the control mice in Table 1, demonstrated a direct response of only 4,490 PFC/spleen (see discussion).

Effect of Thy-1 Administration Schedule

The treatment schedule used for the experiments in Table 1 was chosen because earlier *in vitro* experiments had demonstrated that Thy-1 glycolipid was most effective when added on days 1, 2, or 3 of culture, with maximum suppressive influence when added on day 1 (40). Next, the possibility that the suppression seen *in vivo* (Table 1) could be increased was tested using a treatment regimen which includes giving Thy-1 glycolipid prior to antigen exposure as well as after. $B10C3F_1$ mice were injected with CBA G_{M1} (containing Thy-1.2) in liposome form for 6 consecutive days, beginning 3 days prior to SRBC administration and continuing 2 days after immunization (Table 2). Again the direct anti-SRBC response was suppressed by 60%. The indirect response for the control group was only 38% of that of the direct response (compared to an indirect control response of 98% of the direct PFC response in Experiment 1, Table 1) but was also suppressed by approximately 60% in the G_{M1}-treated group.

TABLE 2

Suppressive effect of Thy-1 *in vivo* on anti-SRBC response when administered in a single regimen, prior to, simultaneous with, and after antigen

Treatment [a]		Adjusted Anti-SRBC PFC/Spleen [b]	
Day 0	Days -3 to +2	Direct PFC	Indirect PFC
SRBC	———	100.0 $\pm$ 17.9 (4490)[c]	38.3 $\pm$ 20.9
SRBC	CBA G_{M1} (+Thy-1.2)	42.3 $\pm$ 2.3*	12.0 $\pm$ 3.4**

(a) Each mouse received 5 x 10^8 SRBC on day 0 and 1 µg G_{M1} (containing Thy-1.2) per day beginning on day -3 (prior to antigen) and continuing daily to and including day 2 (after antigen). G_{M1} was formulated into LF liposomes (see Materials and Methods). The number of PFC/spleen was evaluated 6 days after SRBC injections.

(b) Adjusted mean (see Materials and Methods) $\pm$ std. error for 5 mice/group.

(c) Actual mean PFC/spleen.

Student's t-test (compared to SRBC control) * $P<0.05$ ** $P<0.30$

This finding suggested that increasing the Thy-1 regimen to include pre-exposure of the mice to Thy-1 glycolipid does not increase the suppressive influence of Thy-1.

Further experiments (not shown) were carried out in which the dose of Thy-1 and the administration schedule were varied, including single doses given on various days before, simultaneous with, and after antigen injections. Many of these *in vivo* experiments produced erratic results and did not contribute further to the pool of data.

Thy-1 Enhancement of the Anti-SRBC Humoral Immune Response in Vivo

Thy-1 dose response - liposome morphology. The low control PFC responses seen in Experiment 2, Table 1 (8,430 PFC/spleen), and Table 2 (4,490 PFC/spleen) were thought to be possibly due to the higher levels of antigen being used (5 x 10^8 SRBC) since 10^9 SRBC have been shown to elicit T suppressor activity (43). Accordingly, an SRBC dose response experiment (results not shown) indicated that 5 x 10^7 SRBC would stimulate a higher PFC response in mice than 5 x 10^8 SRBC. It was also suggested that presentation of Thy-1 to the system would be more efficient if Thy-1 were incorporated into small liposomes consisting of a single bilayer rather than large multilamellar liposomes. Since a third preparation of C3H ganglioside which co-migrated with G_{M1} had failed to exert an effect on the PFC response in doses comparable to those used in previous experiments with other G_{M1} preparations, a dose response study was undertaken to compare liposomes containing Thy-1.2 prepared either by low frequency sonication (large multilamellar vesicles, Figure 1a) or high frequency sonication (smaller liposomes of fewer layers,

Figure 1b). B6C3F$_1$ mice were injected with 5 x 10^7 SRBC on day 0, Thy-1 containing liposomes on days 1 and 2, and assayed for anti-SRBC PFC/spleen on day 6. Results are shown in Figure 2.

In contrast to earlier results demonstrating suppressed immune responsiveness, the reverse effect was seen with this preparation of G$_{M1}$ (plus Thy-1). There was an increase in the PFC response of the Thy-1-treated groups over the control group PFC, with a maximum enhancement of over 100% (206 adjusted PFC) in the group receiving 6 µg of C3H G$_{M1}$ in liposomes formed by sonication at 60 Hz (o). Liposomes prepared at 20,000 Hz (●) also elicited an increased response with a peak enhancement of 38% in the group treated with 6 µg, although, in this experiment the enhancement was not significant by Student's t-test.

After adjustment for goat anti-mouse IgG suppression of IgM plaques (see Materials and Methods), there was no measurable IgG response, probably due to the decrease in antigen used.

Initial studies pointed out that Thy-1 was most effective when administered at 24 and 48 hours after immunization with antigen. Thy-1-containing HF liposomes were prepared and administered to B6C3F$_1$ mice in increasing doses as before (o) with the exception of one group which received the Thy-1 dose 6 hours and 30 hours after SRBC injection (●) in an effort to increase the effect of the peak response dose (Figure 2) of 6 µg. A much larger dose of 16 µg was also included in this experiment for comparison with the lower doses. As illustrated in Figure 3, again an enhancement of the PFC response of 30% was noted when the mice were given 6 µg of G$_{M1}$ (plus Thy-1.2) at 24 and 48 hours. Administration at 6 and 30 hours appeared to have increased the enhancement from 30% to

Figure 2. Effect on anti-SRBC PFC of varying doses of Thy-1.2 glycolipid contained in HF and LF liposomes. B6C3F$_1$ (Thy-1.2) mice were injected on day 0 with 5 x 10^7 SRBC followed 24 and 48 hours later by injections of CBA G$_{M1}$ (plus Thy-1.2) in liposome form as described in Materials and Methods. Spleens were assayed for anti-SRBC PFC/spleen on day 6. Each point represents the adjusted mean (see Materials and Methods) ± std. error of 3 mice. Only direct PFC are represented; no indirect PFC were detectable by day 6 in this assay. Actual PFC/spleen for SRBC controls: 60 Hz (o) 27,640 PFC; 20,000 Hz (●), 58,800 PFC.

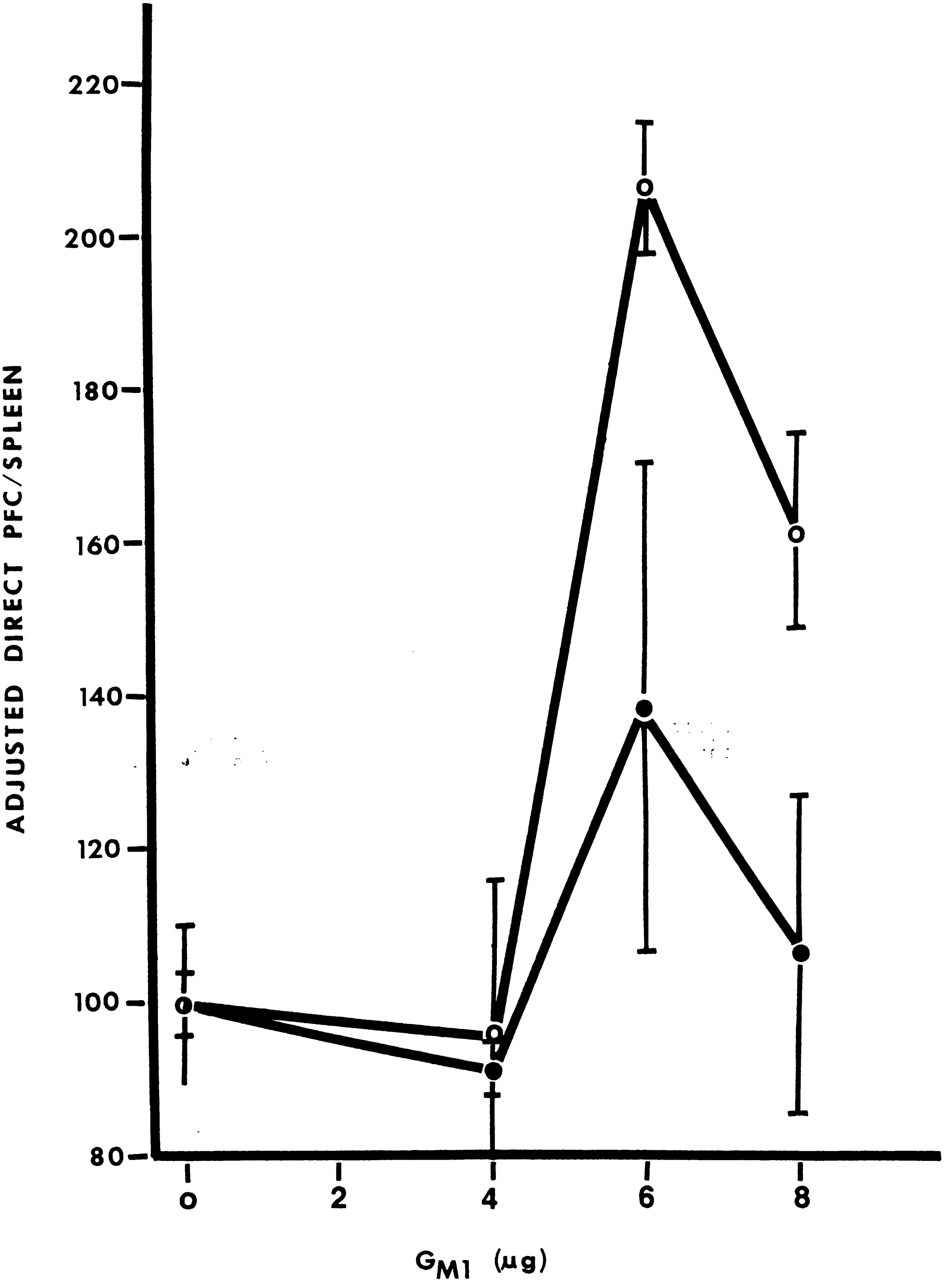

ADJUSTED DIRECT PFC/SPLEEN
220
200
180
160
140
120
100
80
o
2
4
6
8
G_{M1} (µg)

FIGURE 2

Figure 3. Dose response of HF liposomes containing Thy-1.2 glycolipid: effect of shift in post-antigen administration schedule. $B6C3F_1$ mice received 5 x 10^7 SRBC on day 0 and varying doses of liposomes containing C3H G_{M1} (plus Thy-1.2) at 24 and 48 hours followed by assay for number of direct and indirect PFC/spleen on day 6. One group (●) received liposomes containing 6 µg G_{M1} at 6 and 30 hours after antigen. Each point represents the adjusted mean (see Materials and Methods) ± std. error of 4 mice. Values represented are direct PFC only; no indirect PFC were detected by day 6 of this assay. Actual PFC/spleen for SRBC control: 23,100.

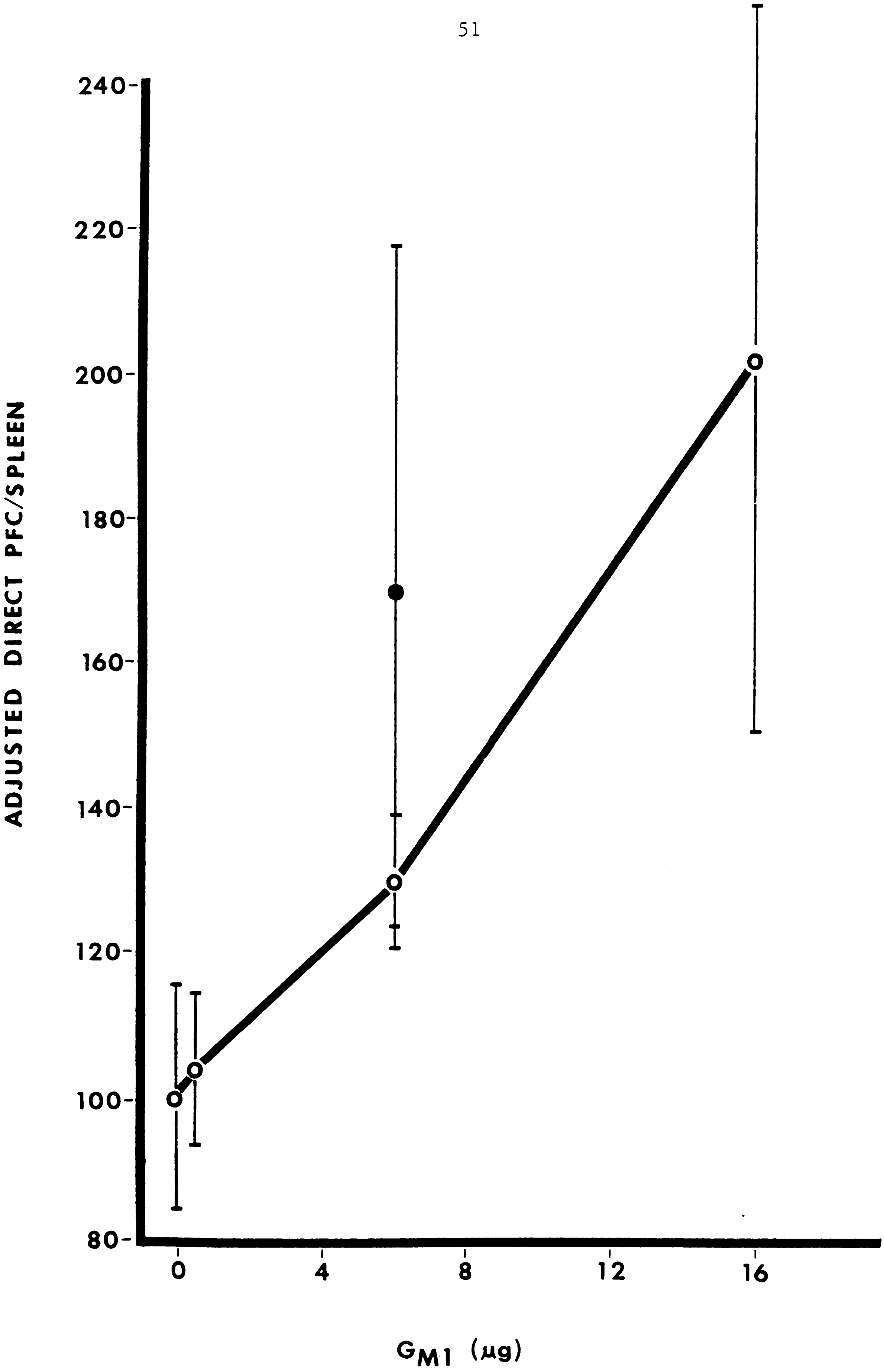

ADJUSTED DIRECT PFC/SPLEEN
240
220
200
180
160
140
120
100
80
0
4
8
12
16
G_{M1} (µg)

FIGURE 3

70%, an increase which is not statistically significant in the experiment. Administration of 16 µg G_{M1}/mouse at 24 and 48 hours increased the response by 100% (203 adjusted PFC/spleen), an increase equal to that of the 6 µg dose of G_{M1} incorporated into LF liposomes. It can be concluded that Thy-1 presentation to the system for a given dose is less effective when prepared in HF liposomes than when administered as LF liposomes, requiring up to 2.5 times more Thy-1 to see a comparable enhancement of the response. Again, using 5 x 10^7 SRBC, there was no significant indirect response when assayed on day 6.

Requirement for liposome form of Thy-1. *In vitro* experiments demonstrated the requirement that Thy-1 be incorporated into liposome form to be effective (49). A comparable experiment was carried out in which B6C3F_1 mice received either cholesterol:lecithin liposomes containing Thy-1 glycolipid, liposomes devoid of Thy-1, or Thy-1 glycolipid in the absence of auxillary lipids, after receiving 5 x 10^7 SRBC as before. HF liposomes were formulated using 15 µg doses of C3H G_{M1} (plus Thy-1.2). Liposome preparation by this method allowed the use of higher doses of Thy-1 which might be important where Thy-1 was administered without auxillary lipids. The results are given in Table 3.

No significant enhancement of the direct PFC response was observed with treatment by the auxillary lipids alone or G_{M1} (plus Thy-1.2) alone. However, an increase of about 50% (150.2 adjusted PFC/spleen *vs* 100.0 adjusted PFC/spleen) was observed when Thy-1 was incorporated into liposomes. Again, no indirect PFC were detected in the experiment by the day of assay.

TABLE 3

Auxillary lipid requirement for enhancement of anti-SRBC response by Thy-1 glycolipid *in vivo*

Treatment [a]		Adjusted Anti-SRBC PFC/Spleen [b]
Day 0	Days 1 and 2	Direct PFC
SRBC	———	100.0 ± 30.9 (46,000)[c]
SRBC	C:L liposomes only	119.1 ± 14.6
SRBC	C3H G_{M1} (plus Thy-1.2) only	96.8 ± 7.7
SRBC	C3H G_{M1} + C:L	150.2 ± 24.8*

(a) B6C3F$_1$ mice received 5 x 10^7 SRBC on day 0. Mice receiving G_{M1} were given 15 µg each on days 1 and 2. Mice given C:L were given 75 µg C, 75 µg L (see Materials and Methods). Liposomes were prepared with high frequency (20,000 Hz) sonication. The number of PFC/spleen was evaluated 6 days after SRBC injection.

(b) Adjusted mean ± std. error for 5 mice/group (see Materials and Methods).

(c) Actual PFC/spleen.

Student's t-test *$P<0.30$.

Kinetics of Thy-1-influenced enhancement in vivo. Preliminary experiments suggested that the effect of Thy-1 treatment could not be measured by the hemolytic plaque technique until day 6 following antigen exposure. The kinetics of this effect were studied in order to determine the duration of the enhancement first seen at day 6 (Figure 4). $B6C3F_1$ mice were injected with 5×10^7 SRBC, followed on days 1 and 2 by HF liposomes containing Thy-1.2. The direct PFC responses for treated and control groups were identical until day 6, when a 35% increase was measured in the Thy-1-treated (69,000 vs 51,000 PFC/spleen). Although the absolute number of cells responding had declined by day 10, the treated group had double the number of responding cells as the control group (24,000 vs 11,000 PFC/spleen). Eleven days after antigen exposure, the direct response of the treated mice had dropped significantly lower than the control group (8,200 and 18,500 PFC/spleen, respectively).

In this study, a low level indirect response was not evident for either group until day 5, declining somewhat by day 6. The response on days 5 (4,300 PFC/spleen) and 6 (540 PFC/spleen) for the control groups represents that of a single mouse in each group, as evidenced by the large standard errors. No indirect response was manifested by any other mouse in the group. In contrast, the Thy-1-treated group showed a significant indirect PFC response on both days 5 and 6, at least 48 hours before the control groups. By day 7, there was a difference greater than ten-fold in the number of indirect plaques/spleen between Thy-1-treated mice and control mice (5,600 vs 480 indirect PFC/spleen). The relative difference decreased with time as the indirect response increased for both groups. By day 10, the enhancement observed in the group treated with Thy-1 was still evident (10,500 vs 5,800 PFC/spleen).

Figure 4. Kinetics of the effect of Thy-1.2 glycolipid on the anti-SRBC response *in vivo*. B6C3F$_1$ (Thy-1.2) mice were immunized i.v. with 5 x 10^7 SRBC on day 0 followed 24 and 48 hours later by 15 µg C3H G_{M1} (plus Thy-1.2) formulated into HF liposomes with auxillary lipids (see Materials and Methods). Hemolytic plaque assays were performed beginning on days 3 through 11, excluding days 8 and 9. Each point represents the mean ± std. error of 5 mice. Control, (————); Thy-1-treated (- - - - -).

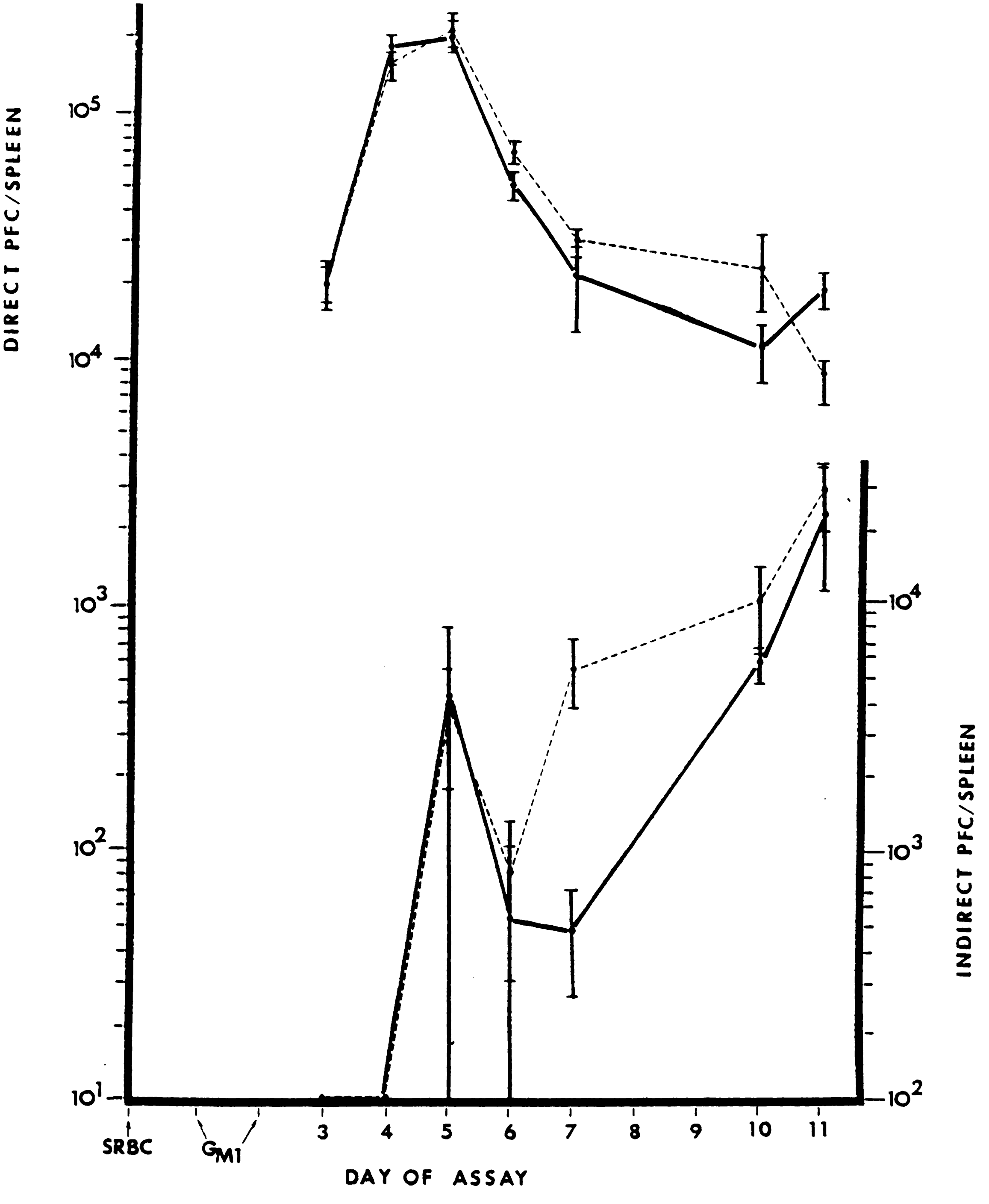

DIRECT PFC/SPLEEN
10^5
10^4
10^3
10^2
10^1
INDIRECT PFC/SPLEEN
10^4
10^3
10^2
SRBC
G_M1
3
4
5
6
7
8
9
10
11
DAY OF ASSAY

FIGURE 4

DISCUSSION

Since previous in vitro studies suggested a regulatory function for Thy-1 glycolipid in the humoral immune response (43), this investigation was undertaken to test further its possible role in the in vivo immune response.

Thy-1 activity has been shown to be associated with gangliosides extracted from mouse brain and thymus in studies on CBA G_{M1}, CBA$_{D1b}$ (both Thy-1.2), and AKR G_{M1} (Thy-1.1) gangliosides (40). Wang et al., (42) were able to show that Thy-1 was only a minor component of the G_{M1} ganglioside preparations. Thy-1 was probably also present as a "contaminant" in the G_{D1b} preparation. When B10C3F$_1$ mice (Thy-1.2) were immunized with SRBC, given CBA G_{M1} (Thy-1.2) intravenously on days 1 and 2, and assayed on day 6 for anti-SRBC PFC, a marked suppression of the response was observed (Tables 1 and 2), in agreement with in vitro results. Mice treated with CBA G_{D1b} also had suppressed PFC responses, while those treated with AKR G_{M1} displayed an apparent, though statistically insignificant suppression in this experiment. The antigenic relationship between Thy-1.1 and Thy-1.2 may be very similar, as heterologous antisera to mouse brain Thy-1.2 does not distinguish between Thy-1.1 and Thy-1.2 in cytotoxicity inhibition studies (29,36,40). Therefore, AKR G_{M1} (Thy-1.1) would be expected to exert a similar, though somewhat less dramatic effect than CBA G_{M1} in this system.

A large disparity was evident between the actual direct PFC/spleen values for SRBC controls in Table 1 (39,900 and 8,430 PFC/spleen) and in Table 2 (4,490 PFC/spleen). This could not be accounted for by differences in the immunocompetence of the mice or the antigenicity of the SRBC due to age differences of the mice or SRBC between experiments since the mice were of similar age and the SRBC were from the same lot for all three experiments. An effort was made to gain some consistancy of the response with respect to the SRBC controls. The use of 5×10^8 SRBC in Tables 1 and 2 represents an antigen dose very near that known to produce suppressor T cells (43) and it is possible that suppressors were being induced by the antigen dose being used, causing the general suppression of the response in all the experimental groups in Experiment 2, Table 1, and Table 2. It was found that a lower dose of SRBC (5×10^7) gave a more consistant response in direct PFC control groups (results not shown). The dose of SRBC was therefore reduced to 5×10^7 SRBC/mouse. In all subsequent experiments, an SRBC control of 23,000 PFC/spleen or greater for the direct response was detected, although the reduced dose failed in most cases to stimulate a measurable indirect PFC response by the sixth day after antigen administration.

Suppression of direct PFC by G_{M1} in Experiment 2, Table 1 was 88% for C3H and CBA G_{M1} (both Thy-1.2) while only 60% in Experiment 1, Table 1, and Table 2. Inconsistancy in the magnitude of the effect exerted by G_{M1} (Thy-1.2) treatment proved to be a problem throughout this study and can only be accounted for by individual differences between mice in each group, due to the many unknown and uncontrollable parameters of an <u>in vivo</u> system. Unfortunately, statistical confidence

was often reduced to 70-80% as a result. Increasing the sample size would probably have increased the statistical confidence in the results, although this was prohibited by the availability of Thy-1 glycolipid, the size of the assay and length of the procedure, and the economics of such an increase. Experiment 1, Table 1 gave a control response in the range average for most experiments (39,900 PFC/spleen). A possible SRBC dilution error resulting in administration of less than 5 x 10^8 SRBC/mouse might account for the difference.

The combination of high antigen dose and Thy-1 treatment might account in part for the increased degree of suppression of the Thy-1-treated groups in Experiment 2, Table 1. It is possible that both antigen-specific and nonspecific suppressor T cells could have been induced by the high SRBC dose. Their effects may have combined additively with the injection of Thy-1, suppressing the immune response by 88%.

In the initial experiments, mice were treated with the gangliosides on days 1 and 2 after SRBC immunization. This protocol was chosen based on _in vitro_ results indicating that maximum suppression was evident when spleen cell cultures were exposed to gangliosides on days 1, 2, or 3 of culture with SRBC (40). In an effort to increase the degree of suppression, several protocols were tested, including exposure to Thy-1 at various times before, simultaneous with, and after antigen exposure. Table 2 illustrates one such attempt to maximize the effect by continuous injections of Thy-1 beginning 3 days before antigen exposure and extending to 2 days after SRBC injection. Maximum suppression was still only 60%, equal to that observed in Experiment 1, Table 1.

The model of Thy-1 modulation of B cell differentiation (43,44) predicts a temporary nonspecific protective suppression of immune responsiveness after initial antigen stimulation until helper factors are available in sufficient concentrations to stimulate B cell proliferation and antibody production. If the model is correct it might be assumed that Thy-1 suppression of the immune response is maximized when the process of Thy-1 shedding by stimulated T cells is artificially prolonged by Thy-1 injections that follow on days 1 and 2.

It was suggested that more efficient presentation of Thy-1 to the reactive cells might be obtained by preparation of the liposomes containing G_{M1} (plus Thy-1) at higher sonication frequencies, reducing the average size of the liposomes and forming a greater proportion of unilamellar liposomes rather than the large multilamellar vesicles formed at the lower sonication frequency (60 Hz) used previously. This would increase the relative liposome surface area available to interact with target cells. Moreover, smaller liposomes are also known to fuse with cell membranes at a much greater rate than large multilamellar liposomes (110). To test this, liposomes were prepared by sonication in 0.85% NaCl at 60 Hz (low frequency - LF; see Figure 1a) and 20,000 Hz (high frequency - HF; see Figure 1b) prior to use. Since preliminary experiments with a fresh preparation of Thy-1.2 glycolipid (contained in C3H G_{M1} ganglioside) had failed to detect any effect using doses of G_{M1} previously used, an experiment was designed to test both the parameters of G_{M1} dose and liposome morphology.

It was hoped that HF sonication would result in a large proportion of unilamellar vesicles of uniform size. Although HF liposomes were smaller on the average than LF liposomes (range 0.02 µm - 0.30 µm vs 0.05 µm - 1.65 µm, respectively), both preparations displayed heterogeneity of morphology. Both LF and HF preparations contained multilamellar liposomes structured of concentric and nonconcentric layers, although these characteristics were more prevalent in LF preparations. The most important result of the HF sonication appeared to be in the reduction of the average size of the liposomes and therefore the G_{M1} (plus Thy-1) content of a given unit. The effect of liposome size on fusion with membranes was not directly investigated in this study.

The experiment designed to investigate G_{M1} dose response of LF and HF liposome preparations was carried out with unexpected results. In direct contrast to earlier results displaying response suppression, the direct (IgM) PFC response was enhanced when measured on day 6 (Figure 2). No indirect (IgG) response was observed due to the low antigen levels used. The maximum relative enhancement was much more dramatic for liposomes prepared at the lower sonication frequency, enhancing the response over the controls by 2.5 times over the enhancement observed for HF liposomes. A disparity in control direct PFC was observed when actual PFC data were studied. Direct PFC/spleen controls gave 58,800 and 27,640 PFC/spleen for the HF and LF liposome preparations, respectively. Enhancement in the former experiment was 22,300 PFC/spleen compared to 29,300 PFC/spleen for the latter, a comparable increase in the actual PFC response. Therefore, while response enhancement was greater for mice treated with LF liposomes relative to controls, the absolute increase was very similar.

The predicted efficiency of Thy-1 presentation to target cells by the smaller liposomes was not observed as an increase in the enhancement of the PFC response of treated animals. It may be that the larger multilamellar LF liposomes are more effective in PFC response modulation because Thy-1 has the effect of being concentrated in discrete units and more Thy-1 can interact with the membrane of a B lymphocyte at a given site. Smaller HF liposomes may fuse more readily with B cell membranes but present much lower doses of Thy-1 at the sites of fusion, thereby failing to elicit the modulatory response. It has been pointed out that large multilamellar liposomes do not recirculate as long as their smaller unilamellar counterparts (111). It may be that a larger proportion are being cleared from circulation in the spleen, releasing more Thy-1 to interact with spleen cells than the more slowly removed smaller liposomes. Less efficient clearing of HF liposomes from the <u>in vivo</u> system may explain why more Thy-1 must be incorporated into HF liposomes to attain a relative effect comparable to that for LF liposomes.

Although the enhancement was reproducible, the degree of the effect continued to be inconsistant (Figure 3). In relative terms, 6 µg G_{M1} incorporated into HF liposomes gave the same approximate degree of enhancement as that in Figure 2 (30% and 38%, respectively), while the actual gain in PFC/spleen by treatment with Thy-1 was only 6,930. When Thy-1 in this form was administered at higher doses (16 µg), enhancement of 100% was obtained, and the actual PFC increase was 23,100 PFC/spleen. A slight change in treatment protocol, administering 6 µg of HF liposomes at 6 and 30 hours rather than the usual 24 and 48 hours did not produce a statistically significant effect for that dose.

That enhancement should be observed with Thy-1 treatment in vivo is not in disagreement with the proposed model of modulation in which both suppression and enhancement are predicted (44). Miller and Esselman reported the peak anti-SRBC PFC response from bone marrow-thymocyte cultures to be at day 11 for the IgM response, followed 24 hours later by the peak IgG response. When treated with medium containing shed Thy-1 from antigen-stimulated T suppressor cells, the responses were at lease 90% suppressed when measured at days 11 and 12 as before. By days 14 and 15, a delay of about 48 hours for each response, the IgM and IgG responses were not only restored, but enhanced by about 30% over the peak responses of the control cultures measured 48 hours earlier. In keeping with these results, the model proposes suppression to be temporary as the shed membrane complex containing Thy-1 renders the B cell unreactive until proper concentrations of T helper molecules accumulate in the microenvironment (44).

Why one preparation of Thy-1 should give enhancement and another suppression in vivo is yet unresolved. This dilemma cannot be explained by the protocol employed. The initial experiments employed very small doses of Thy-1, 1-2 µg G_{M1}/mouse, producing the reduced PFC response (Tables 1 and 2). Later Thy-1 dose response studies with another preparation of G_{M1} (plus Thy-1.2) using equally low quantities were not able to demonstrate suppression (not shown), while raising the dose produced the enhanced PFC response seen in Figures 2 and 3. Very high doses (50 µg) of the original G_{M1} preparations that had suppressed the PFC response had failed to produce the enhancement or increase the suppression (not shown). It is apparent, then, that the disparity is probably not one of

Thy-1 dose. A thin layer chromatography system is now available that will separate Thy-1 from G_{M1} ganglioside (42), increasing its purity. Although it is now known that Thy-1 glycolipid composes a very small fraction of the G_{M1} preparation, a reliable method for quantitating it has not been developed. Eventual accurate quantitation and assessment of purity may help resolve this question. The further possibility that the enhancement might be related to the reduced SRBC dose (5×10^7) was discounted when 5×10^8 SRBC doses did not reverse nor increase the enhancement in Thy-1-treated mice (not shown).

An alternate possibility for the contrasting results may lie in the substrains of mice used for these experiments. The mice used were supposedly of the same strain designated $BC3F_1$ by the suppliers. These were received from two sources: Health Research, Inc., Buffalo, NY (used in the experiments shown in Tables 1 and 2, giving suppression) and Cumberland View Farms, TN (used in all subsequent experiments showing enhancement). Further investigation revealed that $BC3F_1$ mice from Health Research, Inc., were off-spring of the parental genotypes C3H x C57BL/10 Cz, while those from Cumberland View Farms were from the cross C3H x C57BL/6 He. While completely compatible in bone marrow transplant studies (Dr. Harold C. Miller - personal communication), it may be that the genetic differences of these two substrains were responsible for the "opposing" results to treatment with Thy-1 glycolipid. Further studies with both substrains and with other strains of mice with defined genotypes might provide an explanation for this discrepancy. In addition, parallel in vivo and in vitro studies employing a given mouse strain and Thy-1 from the same preparation might provide definitive information concerning the effect seen in vivo compared with that measured in vitro.

In vitro investigations demonstrated the necessity that Thy-1 be incorporated into liposomes with auxillary lipids to demonstrate modulatory activity (40). Enhancement of the antibody response in vivo also required Thy-1 presentation in liposome form (Table 3). Neither G_{M1} (plus Thy-1) nor cholesterol:lecithin liposomes alone showed any effect, but combined, an enhancement in the direct response over controls was observed, an increase of about 23,000 PFC/spleen. In this experiment, HF liposomes (requiring higher G_{M1} content to give a measureable response) were used, allowing a larger dose of G_{M1} to be used in the nonliposome G_{M1}-treated group where it might be important. This provided for a greater probability that an effect would be observed, if Thy-1 alone had the ability to affect the response. No enhancement or suppression was observed for mice treated with G_{M1} in the absence of the auxillary lipids.

The exact role of the auxillary lipids is not known. Vitteta and Uhr (68) demonstrated that surface Ig, selectively shed from lymphocytes in culture, was noncovalently bound to a fragment of the plasma membrane. Further studies indicated that Thy-1 was also selectively shed (58). From their data, these authors proposed a model of membrane elimination in which Thy-1, a peripheral cell membrane antigen, was selectively shed in liposome form (58). Esselman and Miller (43) reported that medium from a T cell culture, prepared from mice primed in vivo with a high dose of antigen (10^9 SRBC) contained Thy-1 activity associated with the lipids extracted from the medium. It may be that Thy-1 is quickly diluted out in vitro and in vivo when introduced in the absence of auxillary lipids, thus becoming ineffective. It is also possible that during isolation, the structure of Thy-1 is altered, leaving its antigenic determinants and active site(s) intact while making it more susceptible to metabolic decay. Association with auxillary lipids may provide temporary protection from metabolic decay while maintaining Thy-1 in discrete concentrated units, increasing efficiency of Thy-1 presentation to target cells. These lipophilic units may also enhance Thy-1 interaction with target cell membranes.

The question of Thy-1 presentation to the in vivo system in liposome form requires further investigation. The release of Thy-1 from viable cells has been documented in vitro in T lymphoma cell lines (39), neuroblastoma cells (Liang Hsu - unpublished) and stimulated T lymphocytes (43,44,55,57). It has been proposed to be shed as a membrane complex in lipsome form (39,43,58). The interaction of membrane-bound Thy-1 with cells in a microenvironment may be distinct from that exerted by Thy-1 bound to an "artificial" liposome such as those used in this study. It is important to note, however, that in vitro studies of Thy-1 function in immune response modulation give support to the functional similarity of shed complexes and cholesterol-lecithin liposomes containing Thy-1. It has been shown that culture medium containing Thy-1 shed from activated T cells (43,44,55) or neuroblastoma cells (Liang Hsu - unpublished) is capable of suppressing the anti-SRBC PFC response in vitro. When Thy-1 glycolipid was extracted from the culture medium by organic solvents and formulated into liposomes with cholesterol and lecithin, suppression of the PFC response was again observed (43,55, Hsu - unpublished). This evidence lends support to the functional similarity of Thy-1 both as a part of a shed complex and an auxillary lipid vesicle.

Antigenic similarity of the extracted Thy-1 molecule with its "intact" counterpart was provided by Wang et al. (42) who demonstrated the ability of the extracted Thy-1 glycolipid to elicit a specific anti-Thy-1 response in a spleen cell culture. Despite the apparent functional and antigenic similarities, it is still possible that subtle structural changes result from the organic solvent extraction that are important to the *in vivo* function of the molecule.

Another important question that needs to be addressed further concerns the similarities of function and antigenic structure of Thy-1 present in the brain tissue and that found on the thymocyte cell surface. It may be that isolated brain associated Thy-1 does not exert the same effects on the immune response that the molecule with the same antigenic specificity isolated from thymic tissue.

The *in vivo* experiments presented here focused on Thy-1 modulation of the humoral immune response by measuring the anti-SRBC PFC/spleen after SRBC immunization and Thy-1 treatment. To maximize the effect in the spleen, both antigen and Thy-1 in liposome form were given intravenously. In a study of liposomes as vehicles for delivery of pharmaceutical agents, Yatvin et al. (111) found an increase in the rate in which the contents of the liposomes were released in the presence of heat-inactivated 10% fetal calf serum. Thy-1-containing liposomes in this system were introduced i.v. and therefore were in contact with serum constituents before interacting with target cells. Serum-liposome interactions constitute a parameter difficult to control or assess and could be responsible for liposome decay and early Thy-1 release or even for the effect that has been observed on the immune response itself.

Artificially raising serum ganglioside levels may represent a phenomenon not known to occur during a normal response to antigen in the spleen. The reaction of Thy-1 with splenic lymphocytes, after entering the spleen via the blood, may be somewhat artifactual. Thy-1 is proposed to be shed by antigenically stimulated T cells and act on B cells in the microenvironment (43). Injection of both antigen and Thy-1 into hind footpad of the experimental animal, allowing both to enter the popliteal lymph node via the lymphatics may provide a more representative model of the *in vivo* effect of Thy-1. While this approach would not necessarily preclude the possibility of liposome interactions with host components, it would increase the possibility of concentrating Thy-1 in the microenvironment of the lymph node, providing more realistic conditions for assessing its *in vivo* effect. These conditions still represent an artificially created state in the lymph node, therefore, the development of a technique to detect natural Thy-1 levels in the germinal centers of the lymph node at various times relative to antigenic stimulation would be important in further study of the *in vivo* role of Thy-1.

A study of the kinetics of the Thy-1 treatment effect showed that enhancement of the direct anti-SRBC PFC response was not evident until the sixth day after antigen exposure (Figure 4). The enhanced response continued until day 10 at almost twice the number of PFC/spleen as controls. In contrast to previous studies using 5×10^7 SRBC, a secondary response was measured for both treated and control groups. On days 5 and 6, the responses for the control group that received only SRBC represented that of only one mouse in each group assayed on those days. Therefore, a significant indirect response for controls was not measured

until day 7 after SRBC injection, while that of the treated group was evident by day 5. Of greater importance was the rapid increase in the IgG response in the Thy-1 treated groups, reaching a level tenfold higher than controls by day 7, a difference which gradually declined as the responses of both groups increased with time.

It seems apparent from both in vitro work and these in vivo studies that the Thy-1 cell surface alloantigen plays a role in modulation of the humoral immune response in the mouse. The model of immunoregulation based on in vitro results (43,44) predicts both a temporary suppressive function for Thy-1 followed by an eventual enhancement of the response. Both effects have been observed here for an in vivo system using the same basic protocol although an explanation is not available. The kinetic studies suggest that the in vivo enhancement is more important as the primary response declines and the secondary response begins to appear. The dramatic effect seen in IgG production poses the possibility that there may also be an effect on the development of immunological memory.

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