SUPPRESSION OF B CELL RESPONSE BY THY-1 GLYCOLIPID SHED FROM NEUROBLASTOMA CELLS

by

Liang Hsu

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1978

ABSTRACT

SUPPRESSION OF B CELL RESPONSE BY THY-1 GLYCOLIPID SHED FROM NEUROBLASTOMA CELLS

by

Liang Hsu

Supernatants from C1300 Neuroblastoma and SaD2 Fibrosarcoma suppressed the anti-SRBC immune responses *in vitro*. This immunosuppression could be reduced by both anti-Thy-1.2 and anti-Thy-1.1 sera absorption pretreatment of tumor supernatant. Immunosuppression was also found in tumor-bearing mice. Evidence for Thy-1 antigen association with the shed material was determined by 1) cytotoxicity inhibition assays whereby both C1300 supernatant and cells could absorb cytotoxicity of anti-Thy-1.2 sera to CBA/J (Thy-1.2) thymocytes, and 2) anti-thymocyte PFC assay in which C1300 supernatant induced *in vitro* formation of specific thymocytotoxic plaques. The molecular nature of Thy-1 shed from C1300 cells was comparable to that shed from lymphoblastoid cells. Ganglioside extracted from C1300 supernatants was found to contain Thy-1 antigenicity, which could also suppress partly anti-SRBC immune response.

Dedicated to my parents and my husband.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my major advisor, Dr. Harold Miller for his valuable guidance and support. I would also like to thank my other committee members, Drs. Walter Esselman, Tobi Jones, and Maria Patterson for their helpful suggestions and discussions.

My sincere thanks go to my dear friends and colleagues, whose willingness to help and encouragement made my graduate study a precious experience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Tumor cells actively shed tumor antigens and membrane complexes which help them to escape from the host's immune response (41,150). The antigen alone or complexes with antibody interfers with the normal regulatory events of lymphocyte interactions that proceed their differentiation to functional, protective states (122,125,116). Gangliosides extracted from mouse brain, thymocytes and supernatant of activated thymocytes have been found to be related to the modulation of anti-SRBC immune response. These glycolipid were shown to be Thy-1 in nature (74,83). Thy-1 shed from two lymphoblastoid cell lines with different allotypes has also been demonstrated to be associated with the suppression of immune response. The suppression induced by Thy-1 ganglioside and shed supernatant of lymphoid cells can be abrogated by anti-Thy-1 sera and anti-G_{M1} sera. The major objective of this research was to investigate the potential for suppression of immune response by supernatant of C1300 neuroblastoma, the presence of Thy-1 antigen on C1300, and Thy-1 ganglioside extracted from C1300 supernatant. The work of others is reviewed to emphasize the shedding of tumor surface components, relation to tumor growth, controversy over the biochemical property of Thy-1 and over the presence of Thy-1 in C1300 cells and the possible biological function of Thy-1.

Suppression of anti-SRBC immune response induced by shed supernatant of neuroblastoma and fibrosarcoma has been found. This immunosuppression could be reduced by preabsorbing tumor supernatant with anti-Thy-1 sera. Thy-1 nature of the material shed from C1300 cells has been established by cytotoxicity inhibition assay in which C1300 supernatant and cells could partially absorb the cytotoxicity of anti-Thy-1

sera to thymocytes, and by anti-Thy-1 PFC assay that thymocytotoxic plaques were induced by shed Thy-1 antigen in C1300 supernatant. The pattern of Thy-1 shedding from C1300 was comparable to that of Thy-1 shed from S49.1 and BW5147 lymphoblastoid cell line. Ganglioside extracted from C1300 supernatants has been found to contain Thy-1 activity, which could also suppress partly anti-SRBC immune response.

LITERATURE REVIEW

Shedding of cell surface components

Phenomenon of shedding

The process of shedding is an event whereby the functional membranes of viable cells release portions of their membrane components or fragments into the extracellular environment, a subject recently reviewed in detail by Dolanski and Kapellar (1). A considerable amount of evidence has emerged supporting the concept of soluble membrane antigens and large membrane complexes shed by both neoplastic (2-6) and normal cells (1,7). Soluble antigens have been found in the surrounding medium (1,8,9) and into the sera of the host (10-14). Large membrane complexes (14-17) which contain specific antigens as well as many undefined membrane proteins and lipids are released, as shed surface Ig (15) and Thy-1 (17), which are bound to a fragment of plasma membrane. The shedding of membrane components is not a random process but occurs at discrete sites on the membrane surface (8,15,17-19). Shedding process is a very selective, regional membrane phenomenon and appears to be a natural event in membrane turnover and elimination (20). Another striking feature of shedding is the apparent enrichment of certain plasma membrane-associated components, but not the general release of plasma membrane (8).

Membrane turnover and kinetics

Initial investigations on membrane turnover were performed by Warren and Glick (20), who followed the kinetics of incorporation of a variety of radioactive precursors into proteins, glycoproteins, glycolipids and phospholipids of membranes isolated *in vitro* from cultured murine fibroblast (L cells). Radiolabelled membrane material was

released into the culture medium from viable cells, suggesting that an active process may be involved in their shedding. Furthermore, metabolic inhibitors significantly slowed the rate of synthesis and led to a concomitant decrease in the rate of degradation, suggesting a coupling of synthesis and degradation. More recently, studies on rates of synthesis and degradation of membrane macromolecules demonstrated that cells actively and continuously synthesize surface membrane to replace those lost by degradation or release (5,6,21-24). Production of various membrane components occurred at different stages of the cell cycle (25). Cikes et al. (26,27) studied asynchronous and synchronous murine lymphoma cells and revealed that H-2 and molony leukemia virus-determined cell surface antigens were maximally expressed in the G_1 growth period. This was inversely related to the growth rate of cells. Lectin binding sites (28) and HL-A antigens (29) on cells were exposed primarily during the G_1 and G_2 phase of the cell cycle. Some complex glycolipids were synthesized during G_1 and early S phase (30). Since these were expressed during G_1 phase, and the antigen was reduced during later stages of the cell cycle, it was possible that shortly after peak expression, these molecules were shed from cell surface.

In most kinetic studies (3,6,21,31,32), biphasic rates of elimination of surface protein and carbohydrates constituents were observed. One occurred during the first 2-5 hours of incubation, while other components turnover at a much slower rate of 2-4 days (3,6,21,31,32). Studies of shedding of specific membrane constituents in both normal lymphocytes and neoplastic cells *in vitro* have also revealed a biphasic release of these macromolecules, as exemplified by shedding of Thy-1 antigen (9,17), H-2 antigen (23), TL antigen from leukemia cell (4),

cell surface Ig from B lymphocytes (15,22) and cell surface glycosyltransferase from fibroblast (33) or neuroblastoma cells (2); events which take place rapidly during the first six hours of incubation in fresh medium. The release of H-2 associated actin in P815 murine mastocytoma was complete within 90 min. Kapeller et al. (6) and Bystryan (5), who followed radiolabelled membrane-associated or-released macromolecules for long incubation periods have observed a slower rate of accumulation of the particular shed components. The quality of these shed materials accumulating in the culture medium during long-term incubation was found to be greater than amounts originally measured on the cell surface (3,4).

Mechanism of shedding

Shedding is a dynamic, energy requiring activity which is an integral part of normal cell metabolism and not the result of cell death or disintegration of the cell membrane into basic components (1). Several observations exclude cell lysis as the source of shedding materials. Warren and Glick (20) demonstrated that metabolic inhibitors reduced membrane turnover. Cone et al. (22) determined that actinomycin A or iodoacetate inhibited the release of cell surface proteins and immunoglobulins from B lymphocytes. Shedding of chicken embryo cell macromolecules was temperature-dependent since reduction to $4^{o}C$ almost completely inhibited shedding (1,6). Koch et al. (8) also found that the shedding process did not occur at a detectable rate if the cells were cooled. Since antiprotease, like soybean trypsin inhibitor (100 µg/ml) and trysylol (50 units/ml), abrogate shedding by 30-50% (6), proteases seem to play a role in shedding process. In addition, cyclohexamide (10 µg/ml) inhibited immediately more than 95% protein synthesis and in 2 hours reduced shedding by 30-50%.

Further evidence (8) indicated that they could not even detect changes in background levels (< 5%) of cell lysis during the shedding, and that the patterns of the protein or glycoprotein product isolated from lysed cells were even more complex than those of membrane preparation. Again confirming that, general cell lysis was inconsistent with the selectivity of shedding.

Investigation of shed material with electron microscopy demonstrated membrane vesicles to be released into surrounding medium, suggesting that these substances are liposomal in nature (34,35). The composition of shed material included all types of membrane constituents (1,20,32). The mechanism of shedding most commonly proposed has been clasmatosis, the pinching off of microvilli (1,8,15,17). After exfoliation (shedding) there was a distinct decrease in the number of microvilli as observed by Normarski inference microscope (8). Many of the microvilli were free in the cultured supernatant, indicating that microvilli have been detached from the cells during the incubation.

Vitteta et al. (17) studied the metabolism of Thy-1 and H-2 in thymocytes, and demonstrated a selective and rapid release of only Thy-1 from the cell surface, suggesting that H-2 antigens were integral protein and Thy-1 as a peripheral molecule. They postulated this to be a regulated release with either H-2 positioned in patches in the membrane so microvilli could pinch off between H-2 antigens or on outer layer of the lipid bilayer pinching off with only peripheral macromolecules, leaving the randomly embedded H-2 antigens on the cell surface. Another discovery which agreed with their contention resulted from work of Walsh and Crompton (36) when they radiolabelled human HLA and Ia antigens on the inner surface of the lipid bilayer of lymphocyte plasma membrane.

The labelled human IgM, mouse IgM, IgD and Thy-1 were detected only on the outer membrane surface. This suggests that cell surface Ig and Thy-1 are peripheral antigens which can be shed. Koch et al. found that shed material, which probably consists of highly purified microvilli of P815 tumor cell lines, contains both actin and H-2. Flanagan (37) also demonstrated that crosslinking of surface Ig by the capping and patching phenomenon induce a specific association between surface Ig and cellular actin. The investigators (8,37) proposed that surface components such as H-2 and Ig can apparently become attached to actin when they are in a polymerized state and since most of membrane-associated actin was localized in the microvilli, the actin may play a role in the pinching off of microvilli.

Biological function of shed material

Shedding of membrane macromolecules has been proposed to play a role in cell replication (38), growth (6), development (39) and differentiation (40). Some shed membrane macromolecules from tumor cells have been postulated to enhance tumor progression (41). It is proposed that shed tumor specific antigen (TSA) and tumor-associated glycoproteins enhance escape from immune destruction by blocking humoral and cellular responses and thus inhibiting direct immunological attack on tumor cells (41-43). There may also be a relationship between the ability of a tumor to metastasize and the rate of spontaneous shedding of surface antigen (41,44).

Thy-1 alloantigen

Definition and distribution

In 1963, Reif and Allen (45) first discovered that the presence of one cell surface alloantigen in AKR/J thymocytes and 1964 (46) demonstrated

that this shared allogenic determinant is in the thymocytes, peripheral T cells and brain cells. Presently, this cell surface antigen is described as Thy-1.1 alloantigen (formerly known as theta antigen), which is one of two allelic forms Thy-1.1 (θ - AKR) and Thy-1.2 (θ - C3H) coded for by the Thy-1 locus on mouse chromosome 9 (47). Thy-1.1 is carried by A-Thy-1^a. AKR/J and a few closely related strains, while Thy-1.2 is carried by all other inbred strains and many wild type mice thus far tested (48). Thy-1 is not a species specific antigen, because a serologically indistinguishable antigen (49), but only one allotypic form Thy-1.1 can be found in rat brain and thymocytes.

Most of the studies to detect the expression of Thy-1 antigen have used cytotoxicity test (45,46,50), cytotoxic inhibition assay (51,52) immunofluorescent techniques (51,53) with either anti-thymocyte antiserum, or heterologous absorbed anti-mouse brain-associated Thy-1 (BA-Thy-1) serum. Thy-1 was found on immature T cells (thymocytes), in high quantity on brain tissues, but rather sparse on peripheral T cells. It also occurs on murine fibroblast cell lines (54), epidermal cells (55), normal and neoplastic mammary cells (56), peripheral and central nervous tissue cells and is detected in much lower concentration in appendix, lung, liver, kidney and neonatal brain (50). Thy-1-like antigen is expressed in brain tissue in rats (57) and possibly in humans (58). It does not occur on normal murine bone marrow cells, B cells (46), plasma cells (59), granulocytes, macrophages, or red blood cells (60). The exact cellular localization and molecular structure remain unknown. In the mouse, membrane bound Thy-1 has been used as a T cell marker and as an example of differentiation antigen due to its distribution and the level of its appearance in murine tissue.

Nature of Thy-1

By different approaches and cell types used to isolate and characterize the Thy-1 antigenic moiety, the biochemical composition of Thy-1 has been reported to be protein (61,62), glycoprotein (63-72) and glycolipids (50,73-75).

(a) Protein nature: Immunoprecipitation of soluble, nondialyzable lysates of radioiodinated cell protein with anti-Thy-1.2 antiserum and excess rabbit anti-mouse Ig serum, Atwell et al. (61) in 1973 reported the nature of Thy-1 antigen on CBA thymocytes (Thy-1.2). Further analysis of the immunoprecipitate by SDS-acryl-amide disc electrophoresis led to the conclusion that Thy-1 was a protein monomer of approximately 60,000 daltons. In 1975, Kucich et al. (62) reported the presence of the Thy-1.2 molecule on one murine lymphoblastoid cell line by cytotoxicity inhibition of anti-Thy-1.2 serum with limited papain or protease-digested-cell supernatant, suggesting that the Thy-1 determinant was protein associated. Thy-1 activity was completely destroyed by prolonged exposure to protease. The size of the material possessing Thy-1 activity was estimated at 200,000 daltons by gel filtration of the concentrated papain digest on sephadex G-200. On the other hand, Letarte-Muirhead et al. (63,64) demonstrated that Thy-1 activity isolated from rat tissue by detergent solubilization was associated with a globular protein of much smaller size, approximately 28,000 daltons. Based on these studies, they suggested that Thy-1 may be protein in nature. But neither preclude that Thy-1 activity was associated with carbohydrates, lipids or glycolipids nature discussed below.

(b) Glycoprotein nature: Some investigators who have studied the Thy-1 of murine thymocyte or rat tissues have concluded that it is a

low molecular weight glycoprotein (63-73). The studies of Letarte-Muirhead et al. (67,68) demonstrated that Thy-1.1 alloantigen from rat thymocytes and brain was a glycoprotein with M.W. 24-28 x 10^3 daltons approximately. They purified deoxycholate solubilized Thy-1 by gel filtration and affinity chromatography on antibody or lentil lectin columns. Chemical analysis of Thy-1.1 (70,72) from rat thymocytes and brain indicated that both Thy-1.1 molecules were glycoproteins containing 30% carbohydrate and with similar amounts of each amino acid but strikingly different in carbohydrate composition. Since they could not detect any antigenic difference between thymocytes and brain Thy-1, they suggested that the antigenic determinants existed on protein. Furthermore, heating and pronase treatment resulted in the loss of Thy-1 antigenic activity. However, other proteolytic enzymes could not effect antigenicity.

Rabbit anti-rat brain serum used to detect three antigenic determinants: Thy-1.1 antigen, rat specific antigen and a crossreactive antigen found on mouse and rat tissue. These three antigen moieties were associated with the same glycoprotein of 28,000 M.W. (71). Arndt et al. (71) have found three antigenic moieties associated with Thy-1 of murine thymocytes. These were all 35,000 daltons, inseparable by serology, gel filtration and isoelectric focusing, suggesting that they were on the same molecule.

Trowbridge et al. (66,76,77) used immunoprecipitation to isolate a glycoprotein of about 25,000 daltons from mouse thymocyte membrane. This molecule was found only on the surface of Thy-1 positive cell line not on derivative Thy-1 negative lines. The material was equally reactive with rabbit anti-thymocyte, anti-mouse T lymphoma and anti-rat

brain-associated Thy-1 sera, suggesting that either this single molecule contained all three antigen specificities or that all the antigenic determinants were identical. The loss of the serologically defined Thy-1 antigenic determinants correlated with the absence of radioactive (H^3-mannose) band corresponding to a T-25 glycoprotein (Thy-1) on the Thy-1 positive cell membrane. Although Thy-1 negative cells did not synthesize T-25, some variants produce a cross-reactive molecule with properties of Thy-1 but containing little carbohydrate. They therefore proposed that Thy-1 antigenicity was retained in the carbohydrate moiety at the terminal sugar residue and Thy-1 negative variants arouse following the loss of glycosyltransferase required to synthesize the presursor of Thy-1 (76). Johnson et al. (78) based their proposal on the fact that neuraminidase, which cleaves sialic acid from suitable substrates, was shown to cause the Thy-1 alloantigen losing its antigenicity. It appears likely that protein may be only partially responsible for the molecule's antigenicity perhaps by serving as a hapten carrier for the carbohydrate moiety.

(c) Glycolipid nature: In 1966, Reif and Allen (45) described the properties of Thy-1 antigen as being nondialyzable and sensitive to lipid solvent. Vitetta et al. (73), in 1973, isolated a complex containing Thy-1 activity from the surface of murine thymocytes and T cells by immunoprecipitating cell lysates of radioiodinated cells with congenic anti-Thy-1 serum. Thy-1 antigenicity could be abolished by treatment of non-ionic detergent NP-40. And all of Thy-1 antigen was sedimented in the lipoprotein region of density gradient. In addition, this entity could be readily labelled with H^3 galactose but not with H^3 amino acids suggesting that a glycolipid moiety was associated with Thy-1 complex.

In 1974, Esselman and Miller (74) proposed that mouse brain ganglioside G_{D1b} was capable of inhibiting the cytotoxicity of anti-brain associated Thy-1 serum when associated with auxiliary lipids. In later studies, Miller and Esselman (75) found that ganglioside from murine brain and thymocytes could absorb the G_{M1} ganglioside from murine brain and thymocytes could absorb the cytotoxicity of anti-Thy-1 serum and to a less extent, rabbit anti-BA Thy-1 serum. A specificity of Thy-1 allotype was also found in G_{M1}, because 2 to 4 fold greater quantities of G_{M1} from AKR/J mice were required to absorb anti-Thy-1.2 cytotoxicity as compared to G_{M1} from Thy-1.2-bearing C3H mice.

In contrast, when Arndt et al. (69) isolated mouse G_{D1b} by a somewhat different method, the material was not capable of absorbing the cytotoxicity of anti-BA-Thy-1 serum. They observed that delipidation of Thy-1 protein fraction by organic solvents caused an 80% loss of original antigenic activity, but the reduced antigenic activity could be restored by the addition of lipids. Therefore, they suggested that thymocyte, and brain Thy-1 antigen was protein in nature, but lipid-protein interaction was necessary for the antigenicity of Thy-1 molecule.

Other studies related to the ganglioside nature of Thy-1 were the following; pretreatment of bone marrow cells or thymocytes (79) with cholerotoxin or choleragenoid, which binds primarily cell surface G_{M1} (80,81), abrogated the cytotoxicity of anti-Thy-1.2 and anti-G_{M1} without affecting anti-H-2 cytotoxicity. Also, pentasaccharide derived from these gangliosides were able to specifically inhibit the appropriate anti-Thy-1 sera in a hapten inhibition assay (82). More recent evidence (83) suggests that the Thy-1 glycolipid was only a small part of G_{M1} and

G_{D1b} preparation used in earlier studies, the specific anti-Thy-1 plaque forming cell assay was used to monitor the improved purification of Thy-1. Thy-1 glycolipid has parallel Thy-1 activity and specificity as Thy-1 glycoprotein.

The co-capping experiments of Thiele et al. (84) also demonstrated that choleragen and anti-Thy-1 displayed a common ligand-induced redistribution, but by immunofluorescence, choleratoxin did not inhibit binding of anti-Thy-1.2, suggesting that the cholera toxin receptor, G_{M1} was closely associated with Thy-1.2, but distinct from any of the antigenic determinants. Immunofluorescent studies by Stein-Douglas et al. (85) demonstrated that binding of anti-G_{M1} to murine thymocytes was not related to Thy-1 allotype.

Immune response to Thy-1

Although mouse strains have the ability to produce antibody against the Thy-1 alloantigen which they lack, qualitative and quantitative variation have been discovered with select genotypic combinations (48). The study of this antibody response led to the development of assays to measure both serum antibody level and quantity of antibody forming cells. Detection of anti-Thy-1 antibodies in the serum of mice immunized with opposite allotypic thymocytes, was tested for the cytolytic ability to thymocytes of the immunizing strains (45,46,50,86). Recently, the method of detecting anti-Thy-1 antibody forming cell assay *in vivo* has been devised by Fuji et al (87) in 1970 and *in vitro* method by Lake in 1976 (88). This assay is a modification of the plaque forming cell (PFC) technique described by Jerne (89). Thymocyte were used as immunogen and target cells *in vivo* system. *In vitro*, shed Thy-1 from thymocytes (88) or lymphoblastoid cells (9) was used as immunogen, and instead of

producing transparent, thymolytic plaques, blue cytotoxic plaques were formed *in vitro* system among a lawn of thymocytes.

The anti-Thy-1 PFC assay was highly specific. Mice that differed in H-2 type and the other cell surface membrane antigen but with identical Thy-1 allotype never showed any significant PFC response at the immunizing dose used (88,90-93). When H-2 histocompatible or congenic mice (differing only in Thy-1 allotype) were used only PFC directed against the immunizing Thy-1 allotype could be detected (93). Another indication of Thy-1 specificity was that only Thy-1 bearing tissue used for immunization could elicit an anti-Thy-1 PFC response, when thymocytes, with highest content of Thy-1, gave the best results as target cells (86,93).

Prerequistes for an efficient murine primary response to Thy-1 antigen have been extensively investigated by Gorzynski and Zaleski (94). It was found that three conditions had to be simultaneously fulfilled in order to produce an optimal response. The responding animal must carry an H-2 complex compatible with that of the donor, especially at the K and D regions. The responding animal must carry a non H-2 background different from that of the donor. Finally, the responding animal need to have T cells reactive to Thy-1.

Extensive genetic studies of anti-Thy-1 response by Zaleski and Klein have led them to propose that the magnitude of anti-Thy-1 PFC response is under the control of one codominant gene, Ir-Thy-1, which is closely linked to the H-2 complex plus at least one minor locus, Ir-5, outside of H-2 complex. By determining the magnitude of primary anti-Thy-1 direct PFC response at six days after the injection of thymocytes, investigators (90,92) classified different strains of mice as

high, intermediate and low responders. A measurable PFC response could be detected after two days of immunization in both low and high responder mice. For low responders, PFC response peaked at 6 days and declined rapidly until there were no detectable PFC at 10 days. While high responder, PFC responses peaked at 4-7 days and lasted until 3 weeks after immunization (48,86,92). The secondary response peaked 3 days later after second injection of thymocytes. In low responders, secondary responses were higher than primary response but in high responder, the primary response was higher. After *in vivo* primary injection, the *in vitro* secondary response peaked at fourth or fifth day of culture (88). IgG PFC were also produced in the secondary response (92) as indicated by determining sensitivity to 2-ME treatment.

Biological significance of Thy-1 alloantigen

Considerable evidence has accumulated suggesting that Thy-1 alloantigen of high concentration is present in murine brain and nervous tissue (46,50). Low amounts of Thy-1 are present in brain at birth, and reach a maximum at about 40 days (50,95). This period coincides with the postnatal development of rodent brain which is highlighted by ongoing myelination and the development of synapse (96). Therefore, Thy-1 may play a role in development. Thy-1 in adult brain is concentrated within synaptosomal fraction (nerve ending) (97) and the temporal occurence of Thy-1 development correlates with synaptogenesis, suggesting that Thy-1 may play an important role in synapse formation (98). Thy-1 is one of the nervous system specific determinants, which may be the means whereby cells identify one another to have cell to cell association (51).

Another possible role of Thy-1 is suggested by Stohl et al. (52)

in the pathogenesis of experimental allergic encephalomyelitis (EAE). Stimulated Thy-1 positive T lymphocyte population in the periphery may cross react with Thy-1 positive elements in central nervous system (CNS) and adversely affect CNS directly.

Since Thy-1 was determined to be a differentiation antigen expressed only on thymus-derived lymphocytes and thymic hormone-treated stem cells, several investigators speculated that Thy-1 may play a role in the regulation of T-cell differentiation or in the immunological function of T lymphocytes (17,99-101). Recent research by Miller and Esselman has demonstrated that a brain and thymic ganglioside with Thy-1 antigenic properties was capable of regulating B lymphocyte antibody responses (33,102,103). Both AKR/J and CBA/J mouse brain G_{M1} ganglioside, once formulated into cholesterol-lecithin liposomes, suppressed anti-SRBC PFC response. This suppression could be abrogated by preabsorption of G_{M1} with anti-Thy-1 alloantiserum. In addition, B lymphocytes were the target of G_{M1} liposomes (75). Similarly, supernatant from T cell culture suppressed antibody response and absorption with anti-Thy-1 or anti-G_{M1} could abrogate the suppression. G_{M1} extracted from suppressor T cell cultures supernatant suppressed anti-SRBC PFC responses and absorbed cytotoxic activity from anti-Thy-1.2 sera (102, 103). It has also been observed that within a few days following peak suppression, the anti-SRBC response gradually returned to normal levels, suggesting that G_{M1} glycolipid temporarily modulate the antibody response. Other studies by Freimuth et al. (9,104) indicated that Thy-1 shed from lymphoblastoid cells was also associated with the suppressive effect to anti-SRBC immune responses. These investigators have proposed that Thy-1 in a glycolipid-liposomal state was shed from T cells and reacts

for a short period of time with B lymphocytes. This interaction prevents direct antigen binding, rendering B cells temporarily unresponsive during the early stages of immune response and protecting them against antigen overloading or tolerance.

C1300 Neuroblastoma

Characteristics

C1300 neuroblastoma is a transplantable tumor of symphathetic nerve cells (105-107). It was the first example of a neuron displaying reversible differentiated features in cell culture. C1300 can grow rapidly in a relatively homogeneous form *in vivo* and *in vitro*. *In vitro*, the tumor cells grow in suspension as anaplastic round cells but certain clone transform to non-dividing forms which extend elongated nerve-like processes when they attach to a surface, and have properties of a mature neuron. These two forms are interconvertible. They can also differentiate *in vivo* when suitable target tissue was used as a stimulus (108). In experimentally induced differentiation of cultured neuroblastoma, various agents including cyclic nucleotides (109,110), anti-metabolites, serum depletion or 5-BUDR treatment (111) have been successfully employed to stimulate growth of neuritic processes. The morphological (110, 112), biochemical (105) and electrophysiological features (113) of these differentiated neuroblastoma are comparable to those of normal, mature neurons. *In vitro*, their doubling time is 17-18 hours (106). C1300-bearing mice usually do not survive after 19-32 days.

Surface antigen

Schachner (114) initially investigated the surface antigen of C1300 cell. She compared the brain cell-specific surface markers representing

central nervous tissue with that of C1300, which is thought to be of peripheral origin. H-2, Thy-1, "NZB" autoantigen (which reacts with autoantibody in NZB mice against thymocytes and brains), Pc-1 (plasma specific antigen) and SK (skin cell-specific antigen) were found on brain tissue. They were also present on C1300 solid tumor cells except lower amount of Thy-1 and "NZB" autoantigen. Mouse species-specific lymphocyte antigen (MSLA) was (115) not found in brain, nor was C1300 cell. However, nerve growth factor S100 and 14-3-2 protein found in soluble extracts of nerve cells were not present in C1300. According to these observations, Schachner proposed that the catalogue of antigens demonstrable on the surface of mouse neuroblastoma C1300, is similar to that of normal brain tissue.

In addition, Gross cell surface antigen (GCSA), which is expressed on cells carrying leukemia virus, is detectable on C1300. No budding C-type particles are seen on the solid tumor but intracisternal A-type particles are plentiful (114).

Thy-1 antigen and C1300 neuroblastoma cells

In 1973, Schachner (114) employed a cytotoxicity inhibition assay with alloantiserum to test Thy-1 expression on C1300 solid tumor and NB41A clonal lines. Some Thy-1 was detected on the solid C1300 tumor cells but its concentration was only about 5% that of the highly Thy-1 positive leukemia RADA1 on a per cell basis. She also excluded the possibility that the low but measurable Thy-1 content was due to T lymphocyte infiltrating the solid tumor. The loss of Thy-1 from the morphologically "differentiated" and "undifferentiated" NB41A clonal cells may be explained by the selection of a Thy-1 negative clone from a genetically heterogeneously stem population, or the prevention of Thy-1

expression by *in vitro* environments since some properties of tumor can be reexpressed again *in vivo*.

Whereas Joseph and Oldstone in 1974, (51) used both congenic serum (A thymocyte injected into A/θ - AKR mouse) and allogenic antiserum for indirect immunofluorescence assay and immunoradioautographic studies but could not detect any Thy-1 antigen in the solid tumor and five clonal lines. Furthermore, by using immunoabsorption test, 25-100 x 10^3 neuroblastoma cells could not absorb 50% of cytolytic activity from test antiserum.

In 1975, Mirky and Thompson (53) employing an immunofluorescence technique with alloantiserum reported the presence of Thy-1 on neuronal-looking cells but not on certain glial cells of cultures of fetal mouse brain. Since fixation of cells prior to the testing gives high autofluorescent background and tends to destroy the antigen, they examined unfixed cultures with immunofluorescence with cells still attached to the culture dish. In this study, they looked directly the expression of antigen on morphologically distinct types of cells and demonstrated that the Thy-1 antigen could develop in culture on the cell surface of different cell types.

Stimulated by this finding, Stohl and Gonatas in 1977 (52) further investigated the presence of Thy-1 on C1300 cells. They used a cytotoxicity inhibition assay with allogenic anti-Thy-1 serum and determine the viability of thymocytes by Chromium^{51} release. Inhibitory capacity by Thy-1 antigen was expressed as the amount of the test material in mg protein needed to remove 50% of the cytotoxicity from the antiserum. C3H whole brain was arbitrarily given a value of 1 and everything else was expressed relative to this in mg protein. Neuroblastoma cells,

trypsinized for 0,1,10 or 30 mim., displayed no detectable Thy-1.

In addition, Zwerner et al. (98) in 1977, employed AKR/J anti-AKR/cum thymocytes serum to detect the presence of Thy-1. After quantitative absorption of anti-Thy-1.2 serum with trypsinized neuroblastoma N-18 clonal cells for 2 hours at $4^{o}C$, absorbed antiserum were tested for the cytotoxic capability to AKR/CUM (Thy-1.2) thymocytes by Cr^{51} release assay. N-18 clonal line failed to express detectable Thy-1.

Soluble immunosuppressive factor released by neoplastic cells

Defective immune responses in tumor-bearing animals (12,41-43,116, 117), or patients (118-120) have been repeatedly reported. Immunosuppressive soluble factors have been found in the serum and ascites fluid of many tumor-bearing animals (41-43,116,121-123). Tumor viruses and their products obtained from infected cells impair a variety of immunological functions (124). The immunosuppressive effects have been proposed as a means of escaping immune surveillance (41). In this way shedding of soluble tumor specific antigens from a variety of neoplastic cells *in vitro* and *in vivo* suppresses lymphocyte differentiation (125), frequency of rosette-forming cells (121,122), graft rejection (118) and PFC responses of spleen cells (116,117,126).

Identification and characterization of tumor immunosuppressive soluble factor have limited studies. Wong et al. (126) found that serum-free supernatant from healthy cultures of non-irradiated fibrosarcoma SaD2 cells were inhibitory at high concentrations. This material was soluble and had a molecular weight of more than 10,000. Yamazak (123) also indicated that the cell-free fluid from Ehrlich ascites carcinoma could suppress the anti-SRBC responses *in vivo*, and the most active fraction had a molecular weight between 1,000 and 10,000. Other factors

described by Chen et al. (127) which were present in ascitic fluid or sera from DMBA-induced thymoma-bearing mice, and caused the decrease of PFC response, were heat sensitive and radiation resistent. The blastogenic lymphocyte response could be either stimulated or inhibited in a dose-dependent manner when tumor cells were added to the cultures. This effect was mediated by a non-dialyzable factor released into the culture medium (128). The immunosuppressive factor from ascites fluid or solubilized cell-free homogenates of mastocytoma (116) described by Kamo et al. was more than 12,000 daltons and heat sensitive (56°C, 30 min.). Primary anti-SRBC immune responses were inhibited by as little as 1% of culture supernatants of L1210 mouse lymphoma cells (117). The factor was a heat labile (56°C, 30 min.) and nondialyzable substance. The target cells were different in various system, for example, helper T cells (129), non-proliferating T cells (122), B cells (130,131).

MATERIALS AND METHODS

Mice

Eight to twelve-week-old C3H x C57BL/6 F1 female mice (Cumberland View Farms, Tenn.) were used in the experiments for detecting the immunosuppression by shed tumor membrane complexes *in vitro*.

For *in vivo* experiments, eleven-to twenty four-week old A/J and thirty-week-old C57BL x DBA/2 (Jackson Laboratory, Bar Harbor, ME) F1 female mice were tested for their immune response to SRBC when bearing Neuroblastoma and Fibrosarcoma respectively.

Nine to twenty-week-old AKR/J and CBA/J female mice were used in the detection of Thy-1 antigenicity *in vitro*.

Usually, five mice matched for age and sex were used in each experimental group.

Tumor cell lines

Neuroblastoma C1300 (Jackson Lab.) is a spontaneous tumor maintained since 1940 by serial transplantation in strain A/J mice. Mice bearing this tumor were obtained from Jackson Laboratories. The solid tumor was adapted to tissue culture conditions by dispersing the cells in Minimal Essential Medium, Hanks Base (Grand Island Biological Co., Grand Island, NY) supplemented with 20% Fetal Calf Serum (FCS) (GIBCO), penicillin-streptomycin (100 unit per ml) and mycostatin (100 unit per ml) (GIBCO).

SaD2 (originating from the Jackson Lab.) is a DBA/2 fibrosarcoma induced by Methylcholanthrene. The cell culture preparation was a generous gift from Dr. Tobi L. Jones (Dept. of Surgery, M.S.U.) cultured in medium CMRL 1066 (GIBCO) with 10% FCS, Pen-Strep and Mycostatin (the

same unit and sources as above).

Both tumor cell lines are maintained at 37°C at 8% CO_2 in humid incubator, hence, cells were trypsinized with 0.1% trypsin (Sigma Chemical Company, St. Louis, MO) in Phosphate Buffer Saline (PBS) with or without Ca^{11} or Mg^{11} and subcultured in the polystyrene tissue culture flask (Falcon Plastics, Los Angeles, CA) when confluent, usually 4 days.

Preparation of spleen cells and thymocytes

Thymus were excised aseptically from AKR/J or CBA/J mice and soaked in Thymocyte medium, which is composed of Dulbeccos' modified MEM (KC Biological Inc., Lenexa, KS) plus 10% FCS, 2 mM Glutamine (GIBCO), Pen-Strep (100 units per ml). Spleens were obtained aseptically from the appropriate strain of mice and soaked in the medium CMRL 1066 (GIBCO) supplemented with 20% FCS, 0.15 mM L-asparagine, 2 mM L-Glutamine, 1 mM Sodium Pyruvate (GIBCO), non-essential amino acids (0.1 mM, GIBCO), Pen-Strep (100 unit per ml), finally neutralized with 7.5% $NaHCO_3$.

Single cell suspensions of splenocytes and thymocytes were prepared by gentle aspiration with a syringe and needles of progressively increasing gauge (21 to 27). After centrifugation at 170 xG ten minutes, cells were resuspended. Viabilities were determined by Trypan Blue exclusion tests and cell concentrations were adjusted at this time.

Antigen

SRBC in Alsever solution (GIBCO) used in the detection of immunosuppression were washed three times in steril PBS, pH-7.2, 1.5% SRBC in PBS were prepared for *in vitro* work. 10^7 SRBC were used *in vivo* studies.

Collection and treatment of tumor supernatant

Neuroblastoma and Fibrosarcoma cells were collected from culture flask by treating with 0.1% trypsin solution, washed and resuspended in its own medium. 2 to 5 x 10^5 viable cells were put in Falcon tissue culture petri dishes and supplied with 5 ml medium. After culturing for different periods of time under optimal growth conditions, the supernatants were collected in plastic tubes and centrifuged at 700 xG for 10 min. at 4^oC to remove any remaining cells, then stored in -40^oC freezer for future use.

Tumor supernatant treated with anti-Thy-1 serum

C3H anti-Thy-1.1 serum and AKR anti-Thy-1.2 serum (Searle Company, High Waycombe, England) with a titer of 1/1,200 and 1/6,000 respectively, which was measured by Cr^{51} release cytotoxicity assay, were being used. Tumor supernatant mixed with anti-Thy-1 serum (with a final dilution 1/500) were stood at 4^oC overnight (16 to 24 hours).

Culture system and assay for detecting immunosuppression by shed tumor material

2 x 10^7 viable spleen cells from BC3F1 mice mixed with an appropriate amount of tumor supernatant or anti-Thy-1 pre-absorbed tumor supernatant and 0.05 ml 1.5% SRBC in a volume of 1.0 ml were placed into the inner compartment of Marbrook vessels, which is separated from a medium reservoir (9.0 ml) by a dialysis membrane. After 5 days at 37^oC in a humid 8% CO_2 atmosphere, the cells from the inner chamber were aspirated. Suspended cells (0.1 ml) were assayed by the Jerne hemolytic plaque method as modified for use with agarose gel on glass microscopic slides (149). In all experiments, the results were

expressed as mean ± standard errors of 5 cultures.

The detection of immunosuppression in tumor-bearing mice

Transplant two groups of A/J mice with appropriate 8 mm^3 amount of solid neuroblastoma at day 0 and day 10. At the 21st day, 10^7 SRBC were injected via the tail vein (control including). Six days later, each spleen was excised and suspended in 1 ml MEM. Single cell suspensions were prepared as above, then 1/10 and 1/100 dilution were made. Both direct and indirect PFC response were measured by counting only the dilution which gave readable numbers of plaques. PFC per spleen and per 10^8 cells were calculated.

Two groups of B6D2F1 female mice were injected subcutaneously in the abdomen with 10^6 SaD2 cells at day 0 and day 10. At the 36th day, 10^7 SRBC were used as antigen. Six days later, assayed as above.

The preabsorption of anti-Thy-1 serum with neuroblastoma cells or supernatants

A two-fold 0.2 ml serial dilution of anti-Thy-1 sera were made and mixed with 0.1 ml prewashed neuroblastoma cells (2 to 3 x 10^7/ml), placed in 37°C waterbath for 30 minutes, refrigerated for at least 2 hours, then centrifuged at 170 xG for 10 minutes. 2.5 ml supernatant were removed to test for their cytotoxicity to thymocytes. Anti-Thy-1 sera not absorbed with C1300 cells through same dilution treatments was used as controls.

A two-fold serial dilution of anti-Thy-1 sera with various amounts of neuroblastoma supernatant were left to stand at 4°C overnight before testing its cytotoxicity to thymocytes.

Cytotoxicity test

Neuroblastoma cell and supernatant-preabsorbed anti-Thy-1 sera were tested for the reduction of titers in the direct cytotoxicity test to thymocytes. 1 to 3 x 10^6 viable thymocytes was suspended in C1300-treated and non-treated anti-Thy-1 sera (total volume 0.8 ml in thymocyte medium) were incubated in 37°C, 8% CO_2 humid incubator for 30 minutes, then 0.15 ml Guinea Pig complement (final dilution 1/30 to 1/40), which has been preabsorbed with agarose (151), were added to each tube. After 45 min., 0.05 ml Trypan Blue (final dilution 0.02%) was added to each tube 5 min. before cell count was made. All other tubes were immersed in the ice to avoid any further reaction. Any blue cells, which had taken up the Trypan Blue dye or damaged cells, which had undergone complete cell lysis, were ignored during the cell count. Therefore, only the transparent, colorless, round viable cells were counted.

Anti-Thy-1 PFC assay

The procedure for induction and assay of the *in vitro* secondary anti-Thy-1 plaque forming cell (PFC) response are modifications of the methods of Fuji et al. (87) and Lake (88) which has been previously described in detail (83).

For studying the secondary response, AKR/J mice were primed by injecting 10^7 CBA/J thymocytes intraveneously at least 7 days before use. Primed CBA mice were prepared similarly but the dose is 4 x 10^7 AKR thymocytes. In some experiments, mice are primed twice before use in order to increase the amount of IgG.

Viable 2 x 10^7 spleen cells (viability greater than 90%) from primed AKR or CBA mice were incubated with equal portions of culture medium and neuroblastoma supernatant (final dilution 1:2 in a volume of

1 ml) or with appropriate amount of fractions from Sepharose-6B column in the inner dialysis compartment of Marbrook vessel. After four days at 37°C in a humid 8% CO_2 atmosphere, viabilities and cell concentrations in each experimental group were measured. Then the cell suspensions in the inner chamber were aspirated and collected into pellets by centrifugation (170 xG) for 5 min. at 4°C.

The spleen cell pellets were resuspended in 0.1 ml of the appropriate target thymocyte suspension containing 2 x 10^7 viable cell (viability greater than 90%) in thymocyte medium (D-MEM/FCS). In some experiments, the cell pellets were mixed with 4 x 10^7 thymocytes, then divided into equal parts for direct and indirect assays. Tubes containing 0.3 ml of 0.6% agarose (Induboise L'Industrie Biologique, Francaise) dissolved in MEM containing 0.5 mg of DEAE-Dextran/ml (Pharmacia Fine Chemicals, Piscataway, NJ) were maintained in a 55°C water bath. The spleen-thymocyte cell suspension was added to the heated agarose solution, vortexed and immediately poured on a microscopic slide previously dipped in a 0.1% agarose solution. After gelation, the slides were turned upsidedown on slide trays; and 1 ml D-MEM/FCS was added to cover each slide before incubating the trays in 37°C humid 8% CO_2 incubator for 5 to 5.5 hours. Each slide was then drained, covered with 15% rabbit complement (lypholized rabbit werum, GIBCO) in D-MEM/FCS and incubated at the same condition for further 45 min. Plaques were determined by a staining technique in which slides were drained and then stained with 0.2% trypan blue in 0.5 M PBS, pH 7.2 for 20 min. at 20°C. Following staining, slides were rinsed twice with PBS and covered with PBS until the dark trypan blue stained plaques were counted under a dissecting microscope adjusted for diffuse illumination. In all

experiments, the results would be expressed as mean ± standard errors of 5 cultures.

Isolation of ganglioside from tumor supernatant

Gangliosides isolated from tumor supernatant by extraction with chloroform:methanol mixtures is a modification of previous method (132). In brief, 50 ml C1300 supernatant was lypholized before extracting with C:M 2:1 mixtures. The extracts were submitted to a Folch partition (133) and the upper ganglioside-rich phase were dried *in vacuo*, subjected to mild alkali hydrolysis with 0.6N NaOH in Meth n l and dialyzed against two changes of distilled water for 48 hours at 4°C. The dialyzed sample was then separated by TLC on silica gel "G" or "60" thin layer plates (E. Merck, Darmstatt, West Germany) with solvent system I (Chloroform - Meth l - 2.5 N NH_4OH - H_2O 60:35:1:7, v/v/v/v) or II (Chloroform - Meth n l - H_2O - $CaCl_2$, 50:40:9:0.1%). Gangliosides were visualized with I_2 vapor and isolated from the TLC plates as described previously (132). The final extracts, which were eluted from silica gel by Chloroform-Meth n l 1:1 and evaporated under nitrogen, were dissolved in 0.5 ml C:M 1:1 mixtures. Gangliosides were prepared for *in vitro* assay by mixing 100 µl ganglioside in C:M 1:1 with 50 µg of lecithin (both obtained from Supelco, Inc., Bellfonte, PA) in C:M 1:1. The mixtures were evaporated under Laminar hood overnight and 1 ml PBS was added followed by sonicating in an ultrasonic cleaner (Mettler Electronic Corp., Anaheim, CA) with 50°C for one min. Each *in vitro* culture received 0.2 ml of this solution.

Gel filtration of C1300 culture supernatant

The neuroblastoma cell free culture supernatants were fractionated

by gel filtration over a sepharose-6B column (1.5 x 60 cm) was equilibrated and run with PBS (pH 7.2). One ml 10-fold concentrated samples, which have been filtered through Amicon CF-50A Centriflo membrane cones (Amicon Corp., Lexington, MA) were applied to the column. Appropriate volumn fractions were tested for their ability to induce a secondary anti-Thy-1.2 PFC response by the usual procedures.

RESULTS

Thy-1 alloantigen shed from two lymphoblastoid cell lines has been found to be associated with the modulation of anti-SRBC immune response (9,104). This may provide an *in situ* mechanism of escape from the host immune system. Thy-1 has been shown to be present on certain other tissues such as brain, nervous tissue, to some extent on skin fibroblasts and other tissues as well. Therefore, two non-lymphoblastoid tumor cell lines, fibroblastoma SaD2 and neuroblastoma C1300 were studied to determine the role of Thy-1 or other glycolipids which may regulate their interactions and immunosuppressive properties.

Immunosuppressive effects of cultured tumor cell supernatants

Various amounts of neuroblastoma C1300 and fibrosarcoma SaD2 supernatant harvested at different times of incubation were added to cultures containing 2 x 10^7 BC3F1 spleen cells, SRBC (1.5%) was used as an antigen. After 5 days, direct PFC response were measured. Results summarized on Table I indicate a decrease of PFC as the time of incubation increased. Comparing results from supernatants of both tumor types harvested at day 1, the PFC response showed little change from the controls; 901 PFC in C1300 supernatant-treated group and 604 PFC in SaD2 supernatant-treated group compared to 1078 PFC and 753 PFC in respective controls. But groups with tumor supernatant from day 3 or day 4 showed the reduction of PFC responses. Further reduction of PFC responses occurred when the amount of tumor supernatant was increased to 0.5 ml of neuroblastoma supernatant from day 4 tumor culture reduced the PFC number from 1078 (group B) to 151 (group H). 0.5 ml supernatant from day 2 cultures further lowered PFC 288 (group G) than that of 0.2 ml (652 PFC, group D). At the time of assay, viabilities and cell numbers

TABLE I

Suppression of antibody response to SRBC by Neuroblastoma and Fibrosarcoma supernatant

Group[a]	Treatment	Anti-SRBC PFC/culture media tested	
		Neuroblastoma	Fibrosarcoma
A	——— without SRBC	150 ± 40[c]	74 ± 16
B	———	1078 ± 236	753 ± 90
C	day 1 supernatant[b] (0.2 ml)	901 ± 110	604 ± 170
D	day 2	652 ± 89	474 ± 57
E	day 3	405 ± 130	389 ± 107
F	day 4	271 ± 63	220 ± 129
G	day 2 supernatant (0.5 ml)	288 ± 116	———
H	day 4	151 ± 36	———

[a]See Materials and Methods for details.

[b]Tumor supernatants were collected as in Materials and Methods.

[c]Means ± Standard errors of 5 cultures per group.

for cultures in each experimental group were measured. No discernible difference in these was indicated when spleen cells were cultured with or without tumor supernatant.

Absorption or immunosuppression by anti-Thy-1 serum

To determine the possible relationship of the shed material with Thy-1 antigen, neuroblastoma supernatant was preabsorbed with predetermined amounts of anti-Thy-1 sera (based on cytotoxic titer) and tested for reduction of immunosuppressive capacity. For these studies, supernatant was mixed with either anti-Thy-1.2 or anti-Thy-1.1 serum for 16-24 hours at $4^{o}C$ before addition to spleen cell cultures.

The PFC responses in groups in which anti-Thy-1.2 or anti-Thy-1.1 preabsorbed C1300 supernatant (Table II) were 397 and 450 PFC respectively compared to 160 PFC in nonabsorbed Group C, the difference was significant at $p \leq 0.05$ confidence level. In other experiments not presented here due to incomplete testing with anti-Thy-1.1, when more anti-Thy-1 serum was used for absorptions, the immune response almost reached the same level as the positive control group. It is important to note that either anti-Thy-1.1 or anti-Thy-1.2 can lower the immunosuppressive effects produced by neuroblastoma supernatant, suggesting that if Thy-1 is related to the immunosuppression, the biological properties may be separate from the antigenic moieties.

In vivo anti-SRBC response in tumor-bearing mice

In attempt to test for suppressive activity *in vivo*, the following experiments were carried out. Fibrosarcoma-bearing BDF1 mice were assayed for their immune response to SRBC. Data presented on Table III show that Group A mice with 42-day fibrosarcoma had approximately 50% decrease

TABLE II

Absorption of suppression in Neuroblastoma supernatant

by anti-Thy-1 serum

Group[a]	Treatment	Anti-SRBC direct PFC/culture [d]
A	——— no SRBC	43 ± 2
B	——— [c]	915 ± 258
C	C1300 supernatant + none (0.5 ml)	160 ± 38
D	+ anti-Thy-1.2[b]	397 ± 98
E	+ anti-Thy-1.1	450 ± 88

[a] Bc3F1 spleen cell cultures with 2 x 10^7 cells in 0.5 ml or 1.0 ml medium were treated with 0.05 ml or 1.5% SRBC in culture.

[b] C1300 supernatant was pretreated with anti-Thy-1 sera (final dilution 1:500) for 16-24 hours at 4°C before addition to spleen cell cultures.

[c] no addition

[d] Statistical data: "C" versus "D", $t=3.01$, $p< 0.02$.
"C" versus "E", $t=2.25$, $p\leq 0.05$.

TABLE III

In vivo anti-SRBC responses in Fibrosarcoma-bearing mice

Group[a]	Days of tumor growth postinoculation before assay	Spleen Cell Number	Anti-SRBC direct PFC $\times 10^2$/spleen	Anti-SRBC direct PFC $\times 10^2/10^8$ spleen cell
A	42	5.22×10^8	164 ± 20[b]	31 ± 4[c]
B	32	1.76×10^8	236 ± 36	134 ± 21
C	0	1.22×10^8	300 ± 72	250 ± 59

[a] BDF1 mice receiving 1×10^6 fibrosarcoma SaD2 cells at day 0 (Group A), day 10 (Group B), were injected with 1×10^7 SRBC at day 36 intravenously, assayed at day 42. Group C were received SRBC only at day 36 and used as a control.

[b] Statistical data: "A" versus "C", $0.05 < p < 0.1$
"B" versus "C", $p < 0.05$

[c] Statistical data: "A" versus "C", $p < 0.05$
"B" versus "C", $p < 0.05$

of PFC response (164 PFC per spleen) compared to that of group C control mice (300 PFC per spleen). There was little difference in PFC responses between group B with 32-day fibrosarcoma and group C.

These tumor cells caused extensive splenomegaly, and it was necessary to rule out the possibility that difference of PFC were due to varying spleen cell numbers among mice. The results were therefore calculated as PFC per 10^8 spleen cells as well as per spleen since the numbers of spleen cells from each mouse were counted at the time of assay. As depicted on Table III and IV, the tumor-bearing mice have 1.4 to 4 times the number of spleen cell that normal mice have (group A and B versus C). By this determination, the PFC in both Group A (3800) and B (13400) mice showed significant decreases ($P < 0.05$) compared to 25000 PFC in Group C.

Neuroblastoma-bearing mice yield similar effects on the immune response. Table IV shows the results of 4 similar experiments. When determined on the basis of PFC response for each individual mouse, i.e. by the unit of PFC per spleen, the anti-SRBC PFC response produced in Group A and B tumor-bearing mice would seem to be not different from Group C control mice. However, by comparison of the PFC response based on the same number of responding cells, i.e. spleen cells, the neuroblastoma-bearing mice showed significantly less PFC ($P < 0.05$).

Thy-1 shedding from neuroblastoma cells

Cytotoxicity inhibition test

Controversy over the presence of the Thy-1 antigen (114,51,52) on neuroblastoma C1300 led us to further examine for its presence in cultured supernatant as well as on cells. This was initially approached using anti-Thy-1 sera which was absorbed with predetermined amounts of

TABLE IV

In vivo anti-SRBC responses in Neuroblastoma

C1300-bearing mice

Group[a]	Days of tumor growth postinoculation before assay	Spleen[b] Cell Number	Anti-SRBC direct PFC[c] x 10^3/spleen	x 10^3/10^8 spleen cell
A	27	4.4×10^8	43.0	9.7
B	17	4.98×10^8	63 ± 14	12.7 ± 0.9
C	0	1.66×10^8	54 ± 13	33 ± 8

[a] A/J mice receiving solid tumor at day 0 (Group A) and day 10 (Group B), were injected with 1×10^7 SRBC at day 21 as well as Group C control, and assayed at day 27.

[b] In group A, the spleen cell numbers was from one surviving mouse. In Groups B and C control, they were the average of spleen cell numbers from 5 mice.

[c] Results from one of 4 similar experiments.
Statistical data: PFC/spleen, "A" versus "C" $p > 0.1$
"B" versus "C" $p > 0.1$
PFC/10^8 spleen cells, "A" versus "C", $p < 0.05$
"B" versus "C", $p < 0.05$

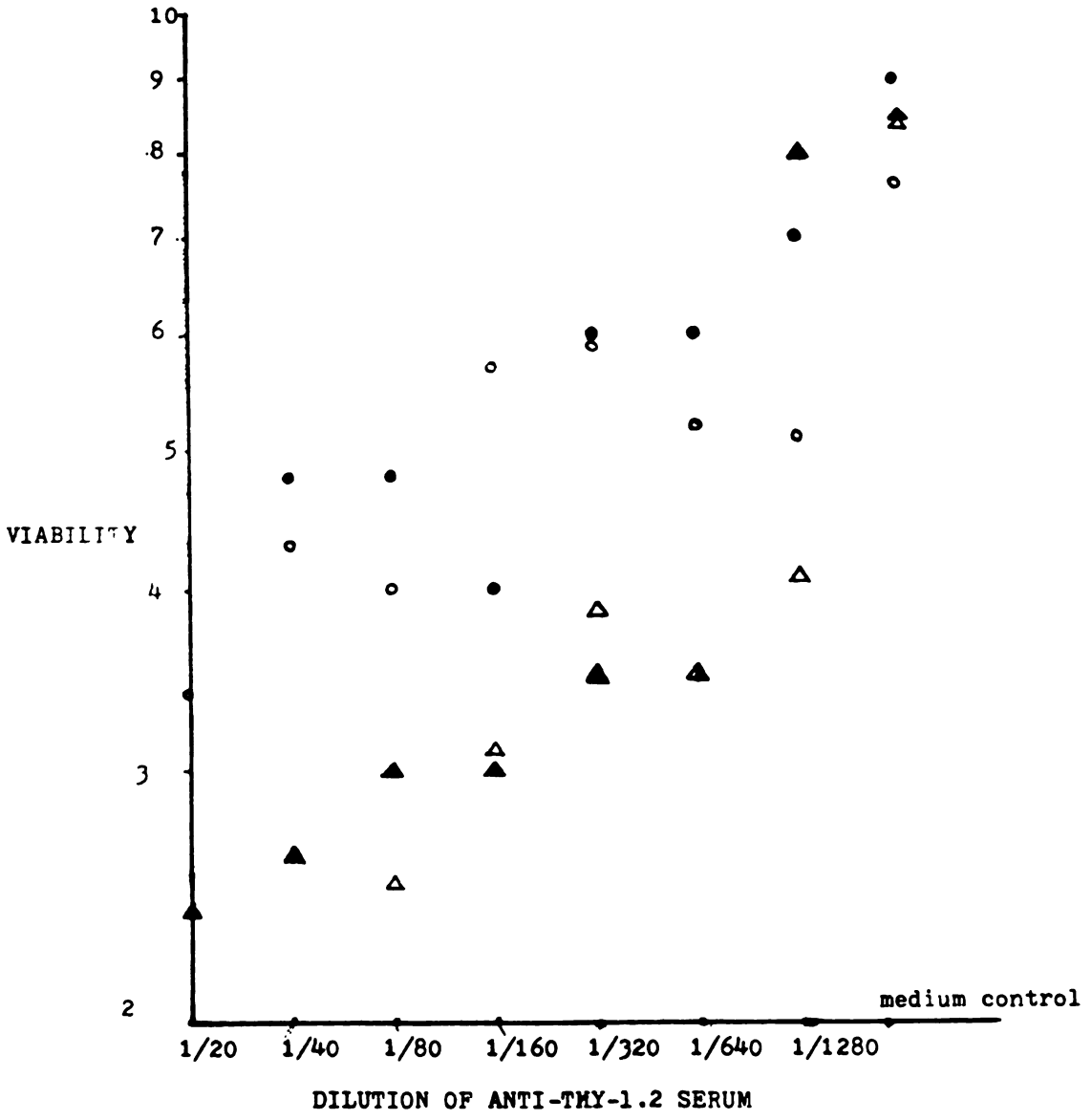

Figure 1. Absorption of anti-Thy-1.2 serum with C1300 supernatants and cells.

either C1300 supernatant or cells, before testing its capacity of killing thymocytes. CBA/J thymocytes (Thy-1.2) was used as target cells.

As the dilution of anti-Thy-1.2 sera was increased, the number of thymocytes killed by antiserum decreased, i.e. viabilities increased. When C1300 supernatant or cells was preabsorbed with serial dilutions of anti-Thy-1.2 serum, higher numbers of viable CBA/J thymocytes were detected compared to non-absorbed antisera, suggesting that anti-Thy-1.2 serum showed lower cytotoxicity. Fresh thymocyte media or cultured C1300 supernatants were used as controls to determine whether normal medium or tumor supernatant plus complement followed by incubation had an effect on viabilities. Viability of these controls was between 76% and 90%. Also, anti-Thy-1.1 did not produce changes in the capacity of killing AKR/J thymocytes (Thy-1.1) after treated with C1300 supernatant (data not presented).

Thy-1 antignicity associated with neuroblastoma supernatant

As a more definitive approach to studying the antigenicity of Thy-1 shed from neuroblastoma cells, anti-Thy-1 responses were followed using primed spleen cells to increase the sensitivity of these assays. In this system, small amounts of antigen were quite effective in inducing a secondary response of 76 PFC per 10^7 spleen cells. Based on previous studies from this laboratory (9), it was determined that S49.1 lymphoid cell lines shed Thy-1.2 and BW5147 cell lines shed Thy-1.1. These shed supernatants therefore provided excellent positive controls for the present studies. AKR/J (Thy-1.1) spleen cell cultures were prepared from AKR/J mice primed with CBA/J thymocytes prior to assays. When neuroblastoma supernatants were used to immunize spleen cell cultures, 117 anti-Thy-1.2 PFC (Table V) were detected after 4 days. This was significantly

TABLE V

Detection of Thy-1 antigenicity in Neuroblastoma supernatant using the thymocytotoxic PFC response

Group	Spleen Cells[a]	Treatment	Target Cells	PFC/10^7 Spleen[b] Cells
				Anti-Thy-1.2
A	AKR/J (1.1)	S49.1 supernatant	CBA/J (1.2) thymocytes	207 ± 10
B		C1300 supernatant + none		117 ± 31
C		+ anti-Thy-1.2		40 ± 4
D				39 ± 5
				Anti-Thy-1.1
A	CBA/J (1.2)	BW5147 supernatant	AKR/J (1.1) thymocytes	75 ± 5
B		C1300 supernatant + none		27 ± 3
C		+ anti-Thy-1.1		29 ± 2
D				31 ± 4

[a]Mice used were primed with thymocytes of opposite Thy-1 allotype before experiments. 2 x 10^7 spleen cells were cultures in 0.5 ml medium and mixed with 0.5 ml tumor supernatant. Anti-Thy-1 pre-absorbed C1300 supernatant were treated in the same way as described earlier.

[b]Results of one out of 3 similar experiments.

different from 38 PFC of background level ($P < 0.001$).

AKR/J thymocytes-primed CBA/J (Thy-1.2) control cultures receiving C1300 supernatant, which were assayed for specific anti-Thy-1.1 secondary response, did not induce PFC over background levels. This suggested that the specificity of Thy-1 allotype in neuroblastoma was maintained. Further confirmation resulted from preabsorption of neuroblastoma supernatant with anti-Thy-1.2 serum, which abrogated the specific anti-Thy-1.2.

Gel filtration fractionation for Thy-1.2 activity

Previous studies concerning the properties of shed Thy-1.2 from our group (9) have shown a peak of the molecular complex to be greater than 2×10^6 daltons. In addition, a smaller quantity of Thy-1.2 was found at the fraction corresponding to 3×10^5 daltons. Gel filtration of neuroblastoma supernatant was carried out to determine if this material was chromatographically comparible to S49.1 lymphoblastoma supernatant.

Supernatant from neuroblastoma cultures was concentrated 10 fold by filtration through Amicon Centriflo membrane cones followed by passage on a Sepharose-6B column to separate shed Thy-1.2 associated complexes. The major absorbance at 280 nm (Figure II) was found in fractions 18-28 and 29-37, which co-chromatographed with bovine serum albumin (M.W. 67,000) and free amino acid respectively. Fractions were chosen to test for their ability to induce an anti-Thy-1.2 PFC response. The fractions inducing significant PFC above background were mainly in fraction 7 and 9, and a smaller quantity in fraction 24. This pattern was comparible to previous discovery that Thy-1 antigenicity was associated with complexes with different molecular weight or properties.

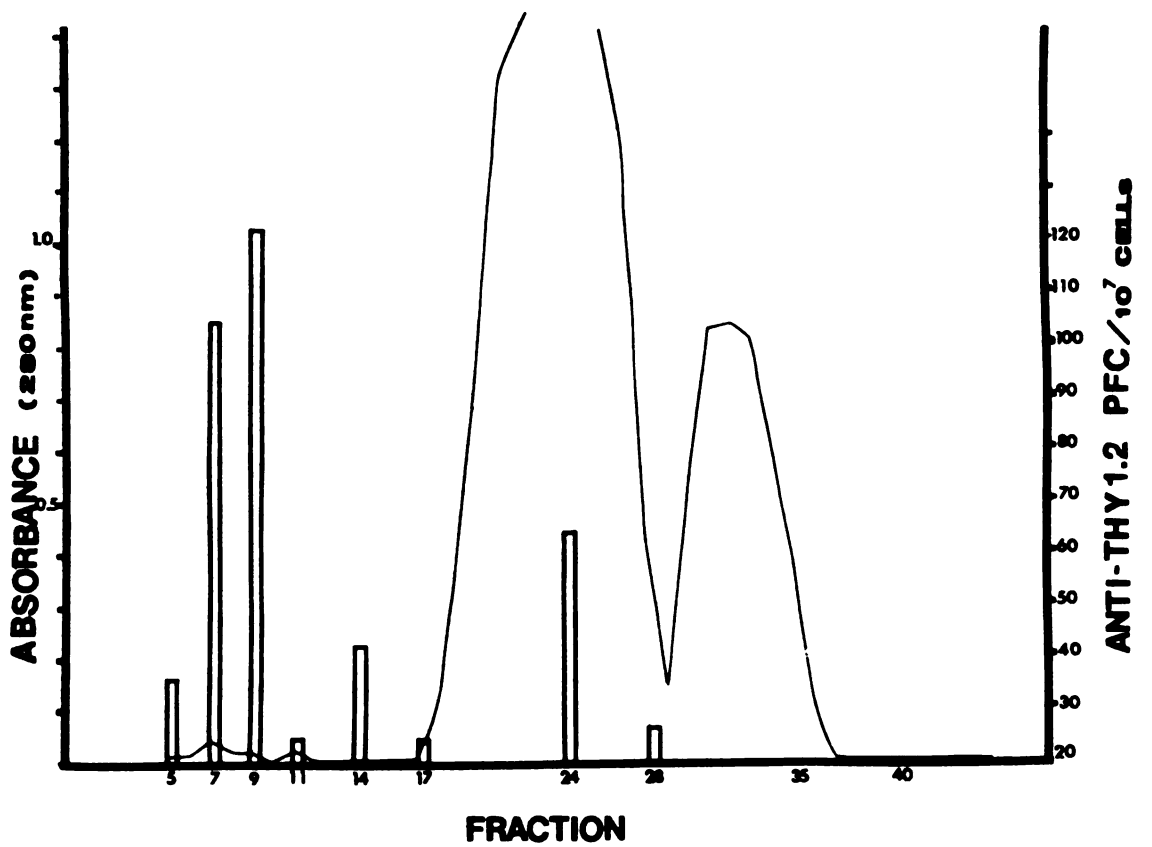

Figure 2. Sepharose-6B fractionation of C1300 supernatant for Thy-1.2 activity.

Determination of Thy-1.2 activity in ganglioside fractions of neuroblastoma supernatant

Ganglioside fractions extracted from mouse brain and thymocytes contain Thy-1 antigen (83). Using different solvents to develop thin layer plates, Thy-1 displayed distinct patterns of motility. In chloroform:methanol:NH_4OH:H_2O solvent, Thy-1 moves just slightly ahead of G_{M1} and G_{D3}, while in chloroform:methanol:H_2O:$CaCl_2$ system, Thy-1 moves between G_{D3} and G_{D1a}, all below G_{M1}.

Ganglioside fractions extracted from neuroblastoma supernatant were developed on thin layer plates using two different solvents. Purified material was added to AKR/J spleen cell cultures to test anti-CBA (Thy-1.2) thymocyte response after 4 days. In the solvent system containing NH_5OH, fraction number 1 migrates slightly ahead of G_{M1} and upon testing yielded 151 plaques, in Table VI compared to the background level 77 PFC, the difference is significant ($P< 0.001$). Similarly, using chloroform:methanol:water system, Group F (fraction 5) showed significantly higher PFC numbers (84 PFC) compared to the background level (30 PFC).

The suppression of anti-SRBC responses by Thy-1 ganglioside

Ganglioside fractions were extracted from neuroblastoma supernatant and prepared with auxilliary lipids (Cholesterol and lecithin), then sonicated to formulate liposomes in PBS and placed in culture with spleen cells. Only the fractions with Thy-1 activity in ammonia solvent (Group C in Table VII) produced more than 50% suppression (2516 PFC in Group C versus 5614 PFC in positive control Group B). Ganglioside fractions from normal C1300 medium in Groups H,I and J demonstrated no significant difference in the PFC responses compared to group B control.

The PFC response of Group G indicated some decrease compared to Group B, but not as much as in group C.

In the water solvent system, fraction 5, which contained Thy-1 suppressive activity as shown above and demonstrated a 40% reduction in anti-SRBC response (3774 PFC in Group G versus 5418 PFC in control Group B). Thus, Thy-1 ganglioside from both systems decreased PFC responses. These results suggest that Thy-1 glycolipid from neuroblastoma supernatant has the capability for suppressing B cell responses, an observation that is consistent with previous studies (75) that Thy-1 glycolipid from mouse brain or thymocytes modulate antibody response and studies on Thy-1 shedding from lymphoma cells (9,104).

TABLE VI

Thy-1.2 activity in ganglioside fraction

of neuroblastoma supernatant

1. Ammonia Solvent System (Chloroform:Methanol:NH_4OH:H_2O)

Group[a]	Ganglioside fraction from C1300 supernatant	Anti-Thy-1.2 direct[b] PFC/10^7 spleen cells
A	———	77 ± 9
B	1	151 ± 7
C	2 (G_{M1})	87 ± 6

[a] AKR/J mice used in this experiment were primed twice with CBA/J thymocytes before experiments, 2 x 10^7 AKR spleen cells in 0.8 ml medium were mixed with 0.2 ml ganglioside in PBS in each culture tube.

[b] Cells in all groups were divided equally with one-half being tested for direct PFC response, the other one-half tested indirectly. "Indirect data are not shown in here. "A" versus "B", $p < 0.001$.

2. Water Solvent System (Chloroform:Methanol:H_2O:$CaCl_2$).

Group[a]	Ganglioside fraction from C1300 supernatant	Anti-Thy-1.2 response[b] PFC/10^7 spleen cells
A	———	30 ± 2.0
B	1	28 ± 1.5
C	2 (G_{M1})	25 ± 1.8
D	3	39 ± 2.1
E	4 (G_{D3})	32 ± 3.9
F	5	84 ± 12.3
G	6 (G_{D1a})	36 ± 3.9

[a] AKR/J mice were primed once. Spleen cell cultures were prepared as above.

[b] "A" versus "D", $p < 0.01$, "A" versus "F", $p < 0.01$.

TABLE VII

Suppression of anti-SRBC response by Thy-1 ganglioside from Neuroblastoma supernatant

1. Ammonia Solvent System:

Group[a]	Ganglioside treatment[b]	Anti-SRBC direct response[d] PFC/culture
A	———— no SRBC	346 ± 69
B	———— [c]	5614 ± 316
C	from C1300 supernatant 1	2516 ± 181
D	2	4048 ± 241
E	3	6080 ± 254
F	4	4278 ± 525
G	from fresh C1300 media 1	3644 ± 384
H	2	4678 ± 663
I	3	5792 ± 385
J	4	5586 ± 507

[a] BC3F1 spleen cell cultures with 2×10^7 cells were treated with 0.05 ml 1.5% SRBC in PBS.

[b] Ganglioside fraction was mixed with cholesterol and lecithin to formulate liposomes, see Materials and Methods for detail.

[c] No addition.

[d] Means ± Standard errors of 5 cultures per group.
Statistical data: "B" versus "C", $p < 0.001$
"B" versus "D" or "G", $p < 0.01$

TABLE VII

(con't)

2. Water Solvent System:

Group[a]	Ganglioside treatment[b]	Anti-SRBC direct response[c] PFC/culture
A	_____ no SRBC	176 ± 61
B	_____	5418 ± 652
C	ganglioside from C1300 supernatant, fraction 1	3892 ± 886
D	2	6094 ± 508
E	3	6446 ± 429
F	4	6210 ± 289
G	5	3774 ± 572
H	6	3448 ± 745
I	7	5022 ± 653

[a,b]Same procedures as above.

[c]Statistical data: "B" versus "G" or "H", $0.05 < p < 0.1$

DISCUSSION

Many tumor cells shed membrane macromolecules or soluble components into the surrounding environment (41,117,121,122,125,133). These have been detected in the serum ascitic fluid or culture medium. Some shed molecules can suppress the immune response as demonstrated not only in the tumor-bearing animals (41,122,125,133) but also cell culture systems (116,121,126). Identification and characterization of immunosuppressive factors produced by tumor cells, however have received only limited attention. Since Thy-1 antigen shed from two lymphoblastoid cell lines has been found to be associated with the suppression of anti-SRBC response (9,104), two non-lymphoblastoid tumor cell lines, neuroblastoma C1300 and fibrosarcoma SaD2, were studied to determine that Thy-1 played a role in their interaction with immune responses.

In this report, significant depression of *in vitro* anti-SRBC plaque forming response was demonstrated by incubation of tumor supernatant from C1300 and SaD2 cell lines with murine spleen cell cultures. Even small volumes of supernatant (0.2 ml) from confluent tumor culture sometimes caused greater than 50% suppression. It is possible that little volume of tumor supernatant was suppressive enough, or contained adequate amounts of immunosuppressive factor. When the amount of supernatant from young tumor culture (not confluent) was increased, further reduction of PFC responses occurred, suggesting that the enrichment of shed material enhanced the suppressive effects. This immunosuppression was not due to depleted nutrients or contamination of tumor supernatant by fungi or bacteria, because cell population and viabilities were similar in both control and supernatant-treated groups after 5 days incubation, also supportedby studies (126,104).

From *in vivo* investigations with SaD2 and C1300-bearing mice somewhat different anti-SRBC responses were found. In SaD2-bearing animals, anti-SRBC PFC responses were lower than those of normal control, while C1300-bearing mice produced higher or similar PFC to those of normal control. Tumor cells induced intensive lymphopoiesis, however, and tumor-bearing mice have enlarged spleen and contain several fold more spleen cells. It was thus necessary to rule out the possibility that differences of PFC were due to varying spleen cell numbers among mice. Upon such recalculation, comparison of experiments and controls based on same numbers of responding cells instead of individual spleens indicated that PFC numbers in C1300-bearing mice were lower than normal control. However, it is possible that if extensive lymphopoiesis was due to response to some tumor specific antigens, the specific immunocompenent clonal lymphocytes against SRBC would be diluted by recalculation. Therefore, the reduction of PFC response resulted from either immunesuppression induced by C1300-tumor supernatant or dilution of immunocompetent cells. Further study is necessary to resolve this matter.

Suppressive activity found in C1300 supernatant was reduced by anti-Thy-1.2 or anti-Thy-1.1 alloantiserum, suggesting that the suppressor molecule may be associated with the Thy-1 antigenic molecule. Both antiserum reduced the immunosuppression induced by C1300 supernatant equally well, which is in line with the observation of previous work (104). The recovery of the PFC response by anti-Thy-1 sera was complete if the suppression in the untreated C1300 supernatant was weak relatively, suggesting the suppressive capacity to be proportional with the amount of Thy-1 antigenic molecule. Normal AKR or C3H mouse sera could not abrogate the suppressive activity as also shown by previous studies

(104), suggesting that anti-Thy-1 sera selectively reacts with the suppressive substance to eliminate its biological function.

Since there is considerable controversy over the presence of Thy-1 on C1300 (51-53,114), further confirmation of the Thy-1 relationship to C1300 was necessary. By direct complement-mediated cytotoxicity test, C1300 cells could not be killed by anti-Thy-1,2 sera. The shedding of Thy-1 has been reported repeatedly from thymocytes and lymphoblastoid cells (9,17). Using cytotoxicity inhibition test, we absorbed anti-Thy-1 sera with both C1300 cells and supernatant could partially reduce the cytotoxicity of anti-Thy-1.2 sera to CBA/J thymocytes but not anti-Thy-1.1 sera to AKR/J thymocytes. C1300 supernatant also showed stronger capacity in absorbing cytotoxicity than C1300 cells, suggesting that Thy-1 antigen in supernatant be more enriched than that on cells. Another approach to detect Thy-1 shedding followed previous work (9,83, 88) by anti-Thy-1 PFC assay. C1300 supernatant could induce significant anti-Thy-1.2 secondary PFC response but not anti-Thy-1.1 PFC response. Anti-Thy-1.2 PFC response was abrogated if C1300 supernatant was pre-treated with anti-Thy-1.2 serum, thus confirming the specificity of shed Thy-1. However, the capacity of C1300 supernatant to induce anti-Thy-1 response was lower than that of S49.1 supernatant. The presence of Thy-1 antigen in C1300 supernatant was not due to cell disintegration or cell death, since supernatant came from cell cultures displaying more than 95% viability.

When C1300 supernatant was passed over Sepharose-6B column, Thy-1.2 antigenicity could be detected in several different fractions. The pattern of Thy-1 activity was comparable to previous work (9) that Thy-1 antigenicity was associated with complexes of different molecular

weight or properties. This raises the possibility that Thy-1 shedding from C1300 represents a mechanism similar to that from S49.1 and BW5147 lymphoblastoid cell lines.

Gangliosides extracted from mouse brain and thymocytes contain Thy-1 activity (74,75,83). We found that gangliosides extracted from C1300 supernatant contained Thy-1 activity no matter what kind of solvents were used to develop thin layer chromatography. The fraction which contained Thy-1 activity could suppress the anti-SRBC immune response as previous finding (74,75). But the immunosuppression induced by C1300 Thy-1 ganglioside was not as strong as that by crude C1300 supernatant. At this point, we are uncertain as to whether inadequate amounts of Thy-1 ganglioside wer used or the possibility that Thy-1 glycoprotein (63-70) might also play a role in modulating anti-SRBC immune response since the process of ganglioside extraction would exclude all protein.

Thy-1 alloantigen as have been defined by cytotoxicity inhibition studies (45,46) and by immunofluorescence techniques (134) with alloantiserum (45,46) or absorbed anti-mouse brain antiserum (135). Thy-1 has a limited distribution among various tissues. It is usually found in high concentration in central nervous system and thymocytes. Differentiated C1300 neuroblastoma cells have many characteristics and properties of mature neuron (105,110,112,113) and their surface antigen components are similar to that of murine brain cells (114). Since brain and neuron cells contain Thy-1 antigen, C1300, a cell line thought to be of symphathetic origin, would be expected to express Thy-1 in some stage of its cycle.

Thy-1, a differentiation antigen, would be expected to be present on maturing or active cells. Usually, differentiation antigens are poorly preserved on cells in culture (136,137). Even in the NB41A clone studied by Schachner (114) was no detectable Thy-1 but it may not actually have lost its original properties during passage *in vitro*. Its expression could be prevented by the *in vitro* environment. Some specific properties of tumors are often re-expressed in the *in vivo* microenvironment (138,139). Thus, the low density of Thy-1 on C1300 may not be unusual.

Previous studies have demonstrated that tumor-specific antigens destroy leukemia lymphocytes but seem harmless or even protective for many carcinoma and sarcoma (140). It has been proposed that target cells for cytotoxicity differ in susceptibility of the surface membrane to lytic attack by activated complement (140). The presence of limited susceptibility areas on the fluid cell membrane, which complement could attack would explain the inability of antibody to lyse the cells (141, 142). The wide distribution of their surface antigen may make it difficult for specific antibodies to activate complement (140). The amount of Thy-1 detected on C1300 was very low compared to concentrated Thy-1 on lymphoblastoid cells (114). Schachner suggested that this could be due to either low amount of Thy-1 on all cells or high amounts on a few cells. It is possible that shedding of Thy-1 from C1300 was very rapid and thus avoided being attacked by activated complement. Previous studies have demonstrated that Thy-1.2 shedding from S49.1 lymphoblastoid cells could be detected after 1 hour incubation with fresh medium (9). In addition, trypsinization was applied to attached C1300 cells before cytotoxicity test but trypsin treatment would inhibit cytolysis of

of Thy-1.2 bearing S49.1 cells by anti-Thy-1.2 sera (78). All of these reasons might explain the incapability of direct cytotoxicity of C1300 cells by anti-Thy-1.2 sera. Those speculations provide some models for explaining the absence of Thy-1 detected by immunofluorescent technique (51). For example, fluorescent anti-mouse Ig plus anti-Thy-1.2 and surface Thy-1.2 may be shed from the surface of C1300 cells so rapidly that they can not be observed by immunofluorescence. If Thy-1 existed in very small amounts on the cell surface of C1300, fluorescent staining by this indirect technique might not be distinguishable from background autofluorescence. The affinity of anti-Thy-1 antibody must also be considered. Akeson and Herschman (143) indicated that specific antigens of nervous system are not detectable by immunofluorescence on "undifferentiated" neuroblastoma cells grown in normal medium but exist on the surface of "differentiated" neuroblastoma cells. In experiments of Joseph et al. (51), the authors concluded that absence of Thy-1 was due to the inability of 25-100 x 10^3 C1300 cells to absorb 50% of cytolytic activity from anti-Thy-o.2 serum. In the studies of Gonatas et al. (52), the absorptive capacity was expressed as the amount of C1300 cells (in mg protein) needed to remove 50% of the cytotoxicity from anti-Thy-1.2 serum. C1300 showed less than 0.05 relative absorption capacity when C3H whole brain was arbitrarily given a value of 1. Much evidence has accumulated to demonstrate that Thy-1 antigenicity is associated with the carbohydrate moiety (66,69,72-74,79) and that although lipid or protein serve as necessary carriers. Thus a presentation of the data in terms of mg protein is not completely valid. Zwerner et al. (98) expressed the inhibition capacity as absorption precentage by C1300 cells on different amount of protein in absorption. The neuroblastoma

clonal line N-18 which they used did show some absorption (less than 20%) but compared to fetal brain, which showed higher than 50% absorption at appropriate amount of protein present in absorption, they denied the presence of Thy-1 in C1300. Data presented in Fig. I were derived from a higher number of C1300 cells used in immunoabsorption than that used by Joseph. C1300 cells-preabsorbed anti-Thy-1.2 sera caused the reduction of cytotoxicity, which is comparable to the finding of Zwerner.

The specificity of the PFC assay for Thy-1 has been established by both *in vivo* immunization of mice with thymocytes (87,91,144) as well as *in vitro* immunization of splenocytes with Thy-1 shed from thymocytes (88) or lymphoblastoid cells (9). Using this same approach, Thy-1 shed from C1300 cells was Thy-1.2 allotype since anti-Thy-1.2 serum pretreatment of C1300 supernatant could abrogate the anti-Thy-1.2 PFC response. C1300 supernatant could not induce any anti-Thy-1.1 PFC above background level. In future, *in vivo* system can be employed for further confirmation of Thy-1 specificity by immunizing A/~~θ~~-AKR mice with C1300 cells or media and then assaying anti-Thy-1.2 PFC response against CBA (Thy-1.2) thymocytes.

Since both anti-Thy-1 alloantisera were effective in neutralizing suppressive activity, it is possible that these were reactive with sites on the released molecules that were separate from the Thy-1 antigenic determinants. Similar phenomena of crossreacting have been observed in previous work, in which suppressive activity found in both S49.1 and BW5147 culture supernatants was abrogated by either anti-Thy-1.1 or anti-Thy-1.2 alloantiserum. It is likely that anti-Thy-1 sera bind directly to the functional portion of the suppressor factor masking this site and preventing its interaction with lymphocyte receptor. There must

be a substance, presumably antibody, in both antisera can react with Thy-1 associated suppressor factor from C1300. Possibly the suppressor factor is represented on a portion of the Thy-1 molecule distinct from the Thy-1 antigenic moiety or on an independent structure associated with it. Thy-1 antigen may be a complex molecule having several different structures associated with it. The antigenic moiety may represent only one part of the whole molecule with the biological function being performed by another site on the molecule. The structural components of Thy-1.1 and Thy-1.2 are so similar that even exhaustively absorbed rabbit-anti-mouse brain associated sera could not detect the difference (135). Experimental evidence has shown that the antigenic moiety of Thy-1 is associated with the carbohydrate portion (66,69,72-74,79). It is possible that Thy-1.1 and Thy-1.2 have same carbohydrate components but different structures (102), in which the difference of sugar sequence, the branching of sugars, the linkage between sialic acid and sugars, or anomerity of monosaccharides can make considerable differences in antigenic nature (145). Thy-1.1 and Thy-1.2 molecule seem to have a common lipid or protein portion (depending on the form studied) as a carrier or backbone of Thy-1 antigenic carbohydrate moiety, which might be analogous to the glycolipid-glycoprotein nature of blood group antigens. The common portion of Thy-1 molecule, supposedly will elicit antibody response as well as antigenic moiety of Thy-1. Therefore, anti-Thy-1 sera may contain heterogeneous antibodies against antigenic carbohydrate, the common backbone, or overlap regions.

Gangliosides extracted from mouse brain and thymocytes contained Thy-1 activity as detected with cytotoxicity inhibition (74,75) or by specific anti-Thy-1 PFC assay (83). G_{M1}, which later on was found to

contain Thy-1 activity could modulate anti-SRBC response through temporarily inhibiting the final stages of B cell differentiation into plasma cells (75,102). Thy-1 in the glycolipid state protects B cells from direct binding and prevents prolonged unresponsiveness due to antigen overload in the early stages of the normal response (75). In addition, it has been recently discovered that the mechanisms of Thy-1 modulation mimic antigen competition (146) again thought to be a modulation state of temporarily blocking B lymphocytes from antigen binding. This *in vitro* observation should also occur *in vivo* but this has been difficult to study. Abundant release of Thy-1 membrane complexes from neoplastic cells may be a continuous process, however, whereby all B cells in localsites of neoplastic cellular changes could be in a permanent modulated state and not able to recover as long as tumor cell shedding of these material continues. Indirect supportive evidences include the finding that Vibrio cholerae neuraminidase (VCN) treatment, which can release sialic acid from both normal and malignant cell surface at physiological pH, of a DBDN-induced fibrosarcoma showed reduced transplantability (147). This is not associated with loss of viability or tumorigenicity but due to increasing immunogenicity. Further support is found in the demonstration that the G_{M1} concentration in the serum of mice or patients with mammary carcinoma (148) was higher than that of normal individuals. Murine G_{M1} ganglioside is structurally related to the Thy-1 antigen (74,75).

In summary, the presence of Thy-1.2 on C1300 cells, and the abrogation of the immunosuppression by anti-Thy-1.1 and anti-Thy-1.2 alloantiserum have demonstrated that shed Thy-1.2 from C1300 supernatant contain suppressive activity to anti-SRBC response. Further extraction of

Thy-1.2 from C1300 supernatant indicated Thy-1,2 glycolipid to be responsible at least in part for immunosuppression. Confirmation has also been found in *in vivo* experiments through demonstration of PFC reduction in tumor-bearing mice. The relation of Thy-1 associated suppressive factor to the total immune surveillance mechanisms and interaction with tumor growth are areas of intense investigation.

BIBLIOGRAPHY

1. Doljanski, F. and M. Kapellar. 1976. Cell surface shedding-the phenomenon and its possible significance. J. Theor. Biol. 62:253.

2. Truding, R. and P. Morell. 1977. Effect of N^6, $O^{2'}$-dibutyryl adenosine 3':5'-monophosphate on the release of surface proteins by murine neuroblastoma cells. J. Biol. Chem. 252;4850.

3. Bystryn, J.C. 1976. Release of tumor-associated antigens by murine melanoma cells. J. Immunol. 116:1302.

4. Yu, A. and E.P. Cohen. 1974. Studies on the effect of specific antiserum on the metabolism of cellular antigen. II. The synthesis and degradation of TL antigens of mouse cells in the presence of TL antiserum. J. Immunol. 112:1296.

5. Bystryn, J.C. 1977. Release of cell surface tumor-associated antigens by viable melanoma cells from humans. J. Natl. Can. Inst. 59:325.

6. Kapellar, M., Y.M. Plesser, N. Kapellar and F. Doljanski. 1976. Turnover and shedding of cell surface constituents in normal and neoplastic chicken cells. In Progress in Differentiation Research. Edited by N. Muller-Berat, North-Holland Publ. Co., Amsterdam, p. 397.

7. Ramseir, H. 1975. Spontaneous release of T-cell receptors for allo-antigens. II. Induction of antibodies to T-cell receptors. Eur. J. Immunol. 5:23.

8. Koch, G.L.E. and M.J. Smith. 1978. An association between actin and the major histocompatibility antigen H-2. Nature 273:274.

9. Freimuth, W.W., W.J. Esselman and H.C. Miller. 1978. Release of Thy-1.2 and Thy-1.1 from lymphblastoid cells: Partial characterization and antigenicity of shed material. J. Immunol. 120:1651.

10. Jehn, W.W., L. Mathanson, R.S. Schwartz and M. Skinner. 1970. *In vitro* lymphocyte stimulation by a soluble antigen from malignant malanoma. New. Eng. J. Med. 283:329.

11. Currie, G.A. and P. Alexander. 1974. Spontaneous shedding of TSTA by viable sarcoma cells: Its possible role in facilitating metastatic spread. Br. J. Cancer 29:72.

12. Currie, G.A. and C. Basham. 1972. Serum mediated inhibition of the immunological reactions of the patient to his own tumor: A possible role for circulating antigen. Br. J. Cancer 26:427.

13. Currie, G.A. and J.O. Gage. 1973. Influence of tumor growth on the evolution of cytotoxic lymphoid cells in rats bearing a spontaneously metastasizing syngenic fibrosarcoms. Br. J. Cancer 28:136.

14. Ben-Sasson, Z., D.W. Weiss and F. Doljanski. 1974. Specific binding of factor(s) released by Rous Sarcoma Virus-transformed cells to splenocytes of chickens with Rous Sarcoma. J. Natl. Can. Inst. 52:405.

15. Vitetta, E.A. and J.W. Uhr, 1972. Cell surface immunoglobulin V. Release from murine splenic lymphocytes. J. Exp. Med. 136:676.

16. Namba, Y. and B.H. Waksman. 1975. Regulatory substances produced by lymphocytes. II Lymphotoxin in the rat. J. Immunol. 115:1018.

17. Vitetta, E.S., J.W. Uhr and E.A. Boyse. 1974. Metabolism of H-2 and Thy-1(θ) alloantigens in murine thymocytes. Eur. J. Immunol. 4:272.

18. Aoki, T. and T. Takahashi. 1972. Viral and cellular surface antigens of murine leukemias and myeloma. Serological analysis by immunoelectron microscopy. J. Exp. Med. 135:443.

19. Marcus, P.I. 1962. Dynamics of surface modification in myxovirus infected cells. Cold Spring Harbor Symp. Quant. Biol. 27:351.

20. Warren, L. and M.C. Glick. 1968. Membranes of animal cells. II. The metabolism and turnover of the surface membrane. J. Cell. Biol. 37:729.

21. Kaplan, J. and M. Moskowita. 1975. Studies on the turnover of plasma membrane in cultured mammalian cells. I. Rate of synthesis and degradation of plasma membrane proteins and carbohydrates. Biochem. Ciophys. Acta 389:290.

22. Cone, R.E., J.J. Marchalonis and R.T. Rolley. 1971. Lymphocyte membrane dynamics: metabolic release of cell surface protein. J. Exp. Med. 134:1373.

23. Kapellar, M., R. Gal-oz, N.B. Grover and F. Doljanski. 1973. natural shedding of carbohydrate-containing macromolecules from cell surfaces. Exp. Cell Res. 79:152.

24. Hudson, J.E. and T.C. Johnson. 1977. Rapidly metabolized glycoproteins in a neuroblastoma cell line. Biochem. Biophys. Acta 497: 567.

25. Bosmann, H.B. and R.A. Winston. 1970. Synthesis of glycoprotein, glycolipid, protein and lipid in synchronized L51781 cells. J. Cell Biol. 45;23.

26. Cikes, M. and G. Klein, 1972. Quantitative studies of antigen expression in cultured murine lymphoma cells. I. Cell-surface antigens in "asynchronous" cultures, J. Natl. Can. Inst. 49: 1599.

27. Cikes, M., S. Friberg and G. Klein. 1972. Quantitative studies of antigen expression in cultures murine lymphoma cells. II. Cell-surface antigens in synchronized cultures. J. Natl. Can. Inst. 49:1607.

28. Fox, T.O., J.R. Sheppard and M.M. Burger. 1971. Cyclic membrane changes in animal cells. Proc. Natl. Acad. Sci. 68:244.

29. Everson, L.K., B.A. Plocinik and G.N. Rogentine, Jr. 1974. HL-A expression on the G1, S and G2 cell-cycle stages of human lymphoid cells. J. Natl. Can. Inst. 53:913.

30. Sakiyama, H. and P.W. Robbins. 1973. The effect of dibutyrl adenosine 3:5'-cyclic monophosphate on the synthesis of glycolipids by normal and transformed NIL cells. Arch. Biochem. Biophys. 154: 407.

31. Melchers, F., R.E. Cone, H. von Boehmer and J. Sprent. 1975. Immunoglobulin turnover in B lymphocyte subpopulations. Eur. J. Immunol. 5:382.

32. Huang, C.C., C.-M. Tsai and E.S. Canellakis. 1973. Iodination of cell membranes. II. Characterization of HeLa cell membrane surface proteins. biochem. Biophys. Acta 332:59.

33. LaMont, J.T., M.T. Gammon and K.J. Isselbacher. 1977. Cell surface glycosyltransferases in cultured fibroblasts: increased activity and release during serum stimulation of growth. Proc. Natl. Acad. Sci. 74:1086.

34. Calafat, J., J. Hilgers, W.J. Van Blitterswijl, M. Verbeet and P.C. Hageman. 1976. Antibody-induced modulation and shedding of mammary tumor virus antigens on the surfaces of GR ascites leukemia cells as compared with normal antigens. J. Natl. Can. Inst. 56:1019.

35. Lutz, H.U., S.-C. Liu and J. Palek. 1977. Release of spectrin-free vesicles from human erythrocytes during ATP deletion. I. Characterization of spectrin-free vesicles. J. Cell Biol. 73:548.

36. Walsh, F.S. and M.J. Crumpton. 1977. Orientation of cell surface antigens in the lipid bilayer of lymphocyte plasma membrane. Nature 269:307.

37. Flanagan, J. and G.L.E. Koch. 1978. Cross-linked surface Ig attaches to actin. Nature 273:278.

38. Rubin, H. 1966. Fact and theory about the cell surface in carcinogenesis. In Major Problems in developmental Biology 25th symposia of the Society for Developmental Biology, M. Locke ed., p.315. Academic Press, New York.

39. Hausman, R.E. and A.A. Moscona. 1973. Cell surface interactions: differential inhibition by proflavone of embryonic cell aggregation and production of specific cell aggregating factor. Proc. Natl. Acad. Sci. 70:3111.

40. Claesson, M.H., S. Whittingham, M.B. Rodger and A.W. Burgess. 1977. Colony growth of human T lymphocyte in agar: Effect of a soluble factor from adherent cells. Eur. J. Immunol. 7:608.

41. Alexander, P. 1974. Escape from immune destruction by the host through shedding of surface antigens: is this a characteristic shared by malignant and embryonic cells? Cancer Res. 34:2077.

42. Baldwin, R., M. Price and R. Robins. 1972. Blocking of lymphocyte mediated cytotoxicity for hepatoma cells by tumor-specific antigen-antibody complexes. Nature New Biol. 238:185.

43. Cooper, A.G., J.F. Copington and M.C. Brown. 1974. *In vivo* release of glycoprotein I from the Ha subline of TA_3 murine tumor in ascites fluid and serum. Proc. Natl. Acad. Sci. 71:1224.

44. Kim, U., A. Baumler, C. Carruthers and K. Bielat. 1975. Immunological escape mechanism in spontaneously metastasizing mammary tumors. Proc. Natl. Acad. Sci. 72:1012.

45. Reif, A.E. and J.M.V. Allen. 1963. Specificity of isoantisera against leukemic and thymic lymphocytes. Nature 200:1332.

46. Reif, A.E. and J.M.V. Allen. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissues. J. Exp. Med. 120:413.

47. Blankenhorn, E.P. and Douglas, T.C. 1972. Location of the gene for theta antigen in the mouse. J. Hered. 63:259.

48. Zaleski, M. and J. Klein. 1978. Genetic control of the immune response to Thy-1 antigens. Immunol. Rev. 38:120.

49. Douglas, T.C. 1972. Occurrence of a θ-like antigen in rats. J. Exp. Med. 136:1054.

50. Reif, A.E. and J.M.V. Allen. 1966. Mouse thymic iso-antigens. Nature 209:521.

51. Joseph, B.S. and M.B.A. Oldstone. 1974. Expression of selected antigens on the surface of cultured neural cells. Brain Res. 80: 421.

52. Stohl, W. and Gonatas, N.K. 1977. Distribution of the Thy-1 antigen in cellular and subcellular fractions of adult mouse brain. 119:422.

53. Mirsky, R. and E.J. Thompson. 1975. Thy-1 (Theta) antigen on the surface of morphologically distinct brain cell types. Cell. 4:95.

54. Stern, P.L. 1973. θ alloantigen on mouse and rat fibroblasts. Nature (New Biol.) 246:76.

55. Scheid, M., E.A. Boyse, E.A. Carswell and L.J. Old. 1972. Serologically demonstrable alloantigens of mouse epidermal cells. J. Exp. Med. 135:938.

56. Gillette, R.W. 1977. Expression of Thy-1 antigen in normal and neoplastic mammary cells of mice. J. Natl. Cancer Inst. 58:1629.

57. Acton, R.T., R.T. Morris and A.F. Williams. 1974. Estimation of the amount and tissue distribution of rat Thy-1.1 antigen. Eur. J. Immunol. 4:598.

58. Ardnt, R., R. Stark and H.G. Thiele. 1977. Detection and molecular characterization of the thymus-brain antigen in human brain. Immunology. 33:101.

59. Raff, M.C. 1969. Theta isoantigen as a marker of thymus derived lymphocytes in mice. Nature. 224:378.

60. Aoki, T., U. Hammerling, E. de Haven, E.A. Boyse and L.J. Old. 1969. Antigenic structure of cell surfaces. J. Exp. Med. 130:979.

61. Atwell, J.L., R.E. Cone and J.J. Marchalonis. 1973. Isolation of theta antigen from the surface of thymus lymphocytes. Nature (New Biol.) 241:251.

62. Kucich, U.N., J.C. Bennett and B.J. Johnson. 1975. The protein nature of the Thy-1.2 alloantigen as expressed by the murine lymphoblastoid line S49.1 TB.2.3. J. Immunol. 115:625.

63. Sauser, D., C. Anckers and C. Bron. 1974. Isolation of mouse thymus-derived lymphocyte specific surface antigens. J. Immunol. 113:617.

64. Letarte-Muirhead, M., R.T. Acton and A.F. Williams. 1974. Preliminary characterization of Thy-1.1 and Ag-B antigens from rat tissues solubilized in detergents. Biochem. J. 14351.

65. Morris, R.J., M. Letarte-Muirhead and A.F. Williams. 1975. Analysis in deoxycholate of three antigenic specificities associated with the rat Thy-1 molecule. Eur. J. Immunol. 5:282.

66. Trowbridge, I.S., I.L. Weissman and M.J. Bevan. 1975. Mouse T cell surface glycoprotein recognized by heterologous anti-thymocyte sera and its relationship to Thy-1 antigen. Nature 256:652.

67. Letarte-Muirhead, M., A.N. Barclay and A.F. Williams. 1975. Purification of the Thy-1 molecule, a major cell-surface glycoprotein of rat thymocytes. Biochem. J. 151:685.

68. Barclay, A.N., M. Letarte-Muirhead and A.F. Williams. 1975. Purification of Thy-1 molecule from rat brain. Biochem. J. 151:699.

69. Arndt, R., R. Stark, P. Klein, A. Muller and H.G. Thiele. 1976. Solubilization and molecular characterization of membrane bound antigens shared by thymocytes and brain. Eur. J. Immunol. 6:333.

70. Barclay, A.N., M. Letarte-Muirhead, A.F. Williams and R.A. Faulkes. 1976. Chemical characterization of the Thy-1 glycoproteins from the membranes of rat thymocytes and brain. Nature. 263:563.

71. Morris, R.J. and A.F. Williams. 1975. Antigens on mouse and rat lymphocytes recognized by rabbit antiserum against rat brain: the quantitative analysis of a xenogeneic antiserum. Eur. J. Immunol. 5:274.

72. Williams, A.F., A.N. Barclay, M. Letarte-Muirhead and A.J. Morris. 1977. Rat Thy-1 antigens from thymus and brain in their tissue distribution, purification and chemical composition. In Cold Spring Harbor Symposia on Quantitative Biology vol. 41, Cold Spring Harbor, N.Y. p. 51.

73. Vitetta, E.S., E.A. Boyse and J.W. Uhr. 1973. Isolation and characterization of a molecular complex containing Thy-1 antigen from the surface of murine thymocytes and T cells. Eur. J. Immunol. 3:446.

74. Esselman, W.J. and H.C. Miller. 1974. Brain and thymic lipid inhibition of anti-brain associated-θ-cytotoxicity. J. E xp. Med. 139:445.

75. Miller, H.C. and W.J. Esselman. 1975. Modulation of the immune response by antigen-reactive lymphocytes after cultivation with gangliosides. J. Immunol. 115:839.

76. Trowbridge, I.S. and C. Mazauskas. 1976. Immunological properties of murine thymus-dependent lymphocyte surface glycoproteins. Eur. J. Immunol. 6:557.

77. Trowbridge, I.S. and R. Hyman. 1975. Thy-1 variants of mouse lymphoma: Biochemical characterization of the genetic defect. Cell. 6:279.

78. Johnson, B.J., U.N. Kucich and A.T. Maurelli. 1976. Studies on the antigenic determinants of the Thy-1.2 alloantigen as expressed by the murine lymphoblastoid line S49.1 TB.2.3. J. Immunol. 116:1669.

79. Milewicz, C., H.C. Miller and W.J. Esselman. 1976. Membrane expression of Thy-1.2 and G_{M1} ganglioside on differentiating T lymphocytes. J. Immunol. 117:1774.

80. Cuatrecasas, P. 1973. Gangliosides and membrane receptors for cholera toxin. Biochemistry. 12:3558.

81. Revesz, T. and M. Greves. 1975. Ligand-induced redistribution of lymphocyte membrane ganglioside G_{M1}. Nature. 257:103.

82. Esselman, W.J. and K. Kato. 1976. Studies on the antigenic nature of murine Thy-1 differentiation antigen. Fed. Proc. (abstr.) 35:1643.

83. Wang, T.J., W.W. Freimuth, H.C. Miller and W. J. Esselman. 1978. Thy-1 antigenicity is associated with glycolipids of brain and thymocytes. J. Immunol. 121:1214.

84. Thiele, H.G., R. Arndt and R. Stark. 1977. Evidence for the presence of choleragen receptor on the thymocyte-brain antigen molecule of mice. Immunol. 32:767.

85. Stein-Douglas, K., G.A. Schwarting, M. Naiki and D.M. Marcus. 1976. Gangliosides as markers for murine lymphocyte subpopulations. J. Exp. Med. 143:822.

86. Fuji, H. M. Zaleski and F. Milgrom. 1972. Genetic control of immune response to θ-AKR alloantigen. J. Immunol. 190:168.

87. Fuji, M., R.T. Schultz and F. Milgrom. 1970. Cytolysis in agar of thumus cells by antibody-forming cells. Proc. Soc. Exp. Biol. Med. 133:180.

88. Lake, P. 1976. Antibody response induced *in vitro* to the cell-surface alloantigen Thy-1. Nature (Lond.) 262:297.

89. Jerne, N.K. and A.K. Nordin. 1963. Plaque formation in agar by single antibody producing cells. Science. 140:405.

90. Zaleski, M.B. 1975. Preliminary evidence of genetic control of the immune response to Thy-1.2 antigen in mice. Immunogenetics 2:21.

91. Zaleski, M.B. 1974. Immune response of mice to Thy-1.1 antigen: studies on congenic lines. Immunogenetics 1:226.

92. Fuji, H., M. Zaleski and F. Milgrom. 1971. Immune response to alloantigens of thymus studied in mice with plaque assay. J. Immunol. 106:56.

93. Fuji, H., M. Zaleski and F. Milgrom. 1971. Allogenic nucleated cells as immunogen and target for plaque-forming cells in mice. Transplant. Proc. III:852.

94. Gorzynski, T. and M. Zaleski. 1978. Prerequisites for an efficient primary response by mice to Thy-1 alloantigens. Lymphocyte recognition mechanisms workshop. The 7th annual meeting of Mid-West Autumn Immunology Conference.

95. Douglas, T.C. 1972. Occurrence of a theta-like antigen in rats. J. Exp. Med. 136:1054.

96. Gonatas, N.K., L. Autilio-Gambetti, P. Gambetti and B. Shafer. 1971. Morphological and biochemical changes in rat synaptosome fractions during neonatal development. J. Cell. Biol. 51:484.

97. Acton, R.T., McClain, L.D. and Zwerner, R.K. 1976. A structural and functional analysis of mouse brain Thy-1. J. Cell Biol. 70:63a.

98. Zwerner, R.K., R.T. Acton and N.W. Seeds. 1977. The developmental appearance of Thy-1 in mouse reaggregating brain cell cultures. Developmental Biol. 60:331.

99. Owen, J.J.T. and M.C. Raff. 1970. Studies on the differentiation of thymus-derived lymphocytes. J. Exp. Med. 132:1216.

100. Raff, M.C. and J.J.T. Owen. 1971. Thymus-derived lymphocytes: their distribution and role in the development of peripheral lymphoid tissues of the mouse. Eur. J. Immunol. 1:27.

101. Scheid, M.P., G. Goldstein, V. Hammerling and E.A. Boyse. 1975. Lymphocyte differentiation from precursor cells *in vitro*. Ann. N.Y. Acad. Sci. 249:531.

102. Miller, H.C., B.J. Laughter and W.J. Esselman. 1977. Regulation of B lymphocytes by T-cell membrane gangliosides. In regulatory mechanisms in lymphocyte activation, edited by D.O. Lucas, Academic Press, New York.

103. Esselman, W.J. and H.C. Miller. 1977. Modulation of B cell responses by glycolipid released from antigen stimulated T cells. J. Immunol. 119:1994.

104. Freimuth, W.W., H.C. Miller and W.J. Esselman. 1978. Soluble factors containing Thy-1 antigen shed from lymphoblastoid cells modulate *in vitro* plaque forming cell response. (Submitted for publication).

105. Augusti-Tocco, G. and G. Sato. 1969. Establishment of functional clonal lines of neurons from mouse neuroblastoma. Proc. Natl. Acad. Sci. U.S.A. 64:311.

106. Schubert, D., S. Humphreys, C. Baroni and M. Cohn. 1969. *In vitro* differentiation of a mouse neuroblastoma. Proc. Natl. Acad. Sci. U.S.A. 64:316.

107. Nelson, P., W. Ruffner and M. Nirenberg. 1969. Neuronal tumor cells with excitable membranes grown *in vitro*. Proc. U.S. Natl. Acad. Sci. 64;1004.

108. Hsu, L. and G.L. Trupin. 1978. *In vivo* differentiation of murine neuroblastoma. Virchows Arch. B Cell Patho. 27:49.

109. Furmanski, P., D.J. Silverman and M. Lupin. 1971. Expression of differentiated functions in mouse neuroblastoma mediated by dibutyrl-cyclic adenosine monophosphate. Nature 233:413.

110. Prasad, K.N. and A.W. Hsie. 1971. Morphological differentiation of mouse neuroblastoma cells induced *in vitro* by dibutyryl adenosine 3" -5' -cyclic monophosphate. Nature 233:141.

111. Schubert, D., S. Humphreys, F. De Vitry and F. Jacob. 1971. Induced differentiation of a neuroblastoma. Dev. Biol. 25:514.

112. Ross, J., J.B. Olmsted and L. Rosenbaum. 1975. The ultrastructure of mouse neuroblastoma cells in tissue culture. Tiss. Cell. 1:107.

113. Nelson, P.G., J.H. Peacock, T. Amano and J. Minna. 1971. Electrogenesis in mouse neuroblastoma cells *in vitro*. J. Cell. Physio. 77:337.

114. Schachner, M. 1973. Serologically demonstrable cell surface specificities on mouse neuroblastoma C1300. Nature New Biol. 243: 117.

115. Shigeno, N., U.C. Hammerling, E.A. Boyse and L.J. Old. 1968. Preparation of lymphocyte-specific antibody from anti-lymphocyte serum. Lancet ii:320.

116. Kama, I., C. Patel, J. Kately and H. Friedman. 1975. Immunosuppression induced *in vitro* by mastocytoma tumor cells and cell free-extracts. J. Immunol. 114:1749.

117. Huget, R.P., H.D. Flad and H.G. Opitz. 1977. Suppression of *in vitro* primary immune response by L1212 cells and their culture supernatant: evidence for cytotoxic effects. Cell. Immunol. 29:210.

118. Gardner, R.J. and F.W. Preston. 1962. Prolonged skin hemograft survival in advanced cancer and cirrhosis of the liver. Surg. Gyn. Obstet. 115:399.

119. Lee, A.K.Y., M. Rowley and J.R. Mackay. 1970. Antibody producing capacity in human cancer. Brit. J. Cancer. 24;454.

120. Al-Sarraf, M., P. Wong, S.Sardesai and V.K. Vairkevisus. 1970. Clinical immunologic responsiveness in malignant disease. I. Delayed hypersensitivity reaction and the effect of cytotoxic drugs. Cancer 26:262.

121. Takada, A., Y. Takada and J. Minowada. 1974. Immunological functions of human T-lymphoid cell line (MOLT). I. Release of immunosuppressive factors from the mixture of MOLT-4 cells and sheep red blood cells. J. Exp. Med. 140:538.

122. Pikovski, M.A., Y. Ziffroni-Gallon and J.P. Witz. 1975. Suppression of immune response to sheep red blood cells in mice treated with preparations of tumor cell component and in tumor-bearing mice. Eur. J. Immunol. 5:447.

123. Yamazaki, H., K. Nitta and H. Umezawa. 1973. Immunosuppression induced with cell-free fluid of Ehrlich carcinoma ascites and its fractions. Gann. 64:83.

124. Dent, P.B. 1972. Immunosuppression by oncogenic viruses. Progr. Med. Virol. 14:1.

125. Gorczynski, R.M., D.G. Kilbum, R.A. Knight, C. Norbury, D.C. Parker and J.B. Smith. 1975. Nonspecific and specific immunosuppression in tumor-bearing mice by soluble immune complexes. Nature 254:141.

126. Wong, A., R. Mankovitz and J.C. Kennedy. 1974. Immunosuppressive and immunostimulatory factors produced by malignant cells *in vitro*. Internat. J. Cancer 13:530.

127. Chan, P.L. and N.R.S.C. Sinclair. 1972. Immunologic and virologic properties of chemically induced and r-irradiation-induced thymic lymphoma in mice. J. Nat. Cancer Inst. 48:1629.

128. Anderson, R.J., C.M. Mebribe and E.M. Hersh. 1972. Lymphocyte blastogenic response to cultured allogenic tumor cells *in vitro*. Cancer Res. 32:988.

129. Kamo, I., C. Patel, N. Patel and H. Friedman. 1975. Restoration of *in vitro* immune responsiveness of mastocytoma-suppressed splenocytes by activated T cells. J. Immunol. 115:382.

130. Friedman, W.H., A. Guimezanes and R.H. Gisler. 1977. Characterization of suppressive immunoglobulin-binding factor. I. Production of IBF by a θ-positive lymphoma (L-5178-Y). J. Immunol. 119:1266.

131. Neauport-Sautes, C., and W.H. Friedman. 1977. Characterization of suppressive immunoglobulin-binding factor (IBF). II. Purification and molecular weight determination of IBF produced by L-5178-Y θ-positive lymphoma. J. Immunol. 119:1269.

132. Esselman, W.J., R.A. Laine and C.C. Sweely. 1972. Isolation and characterization of glycolipids, in Methods in Enzymology (V. Ginsbury. ed.) Academic Press, N.Y. 28:140.

133. Folch, J.M., M. Lees and G.H. Sloane Stanely. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497.

134. Raff, M.C. 1970. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. Immunology. 19:637.

135. Golub, E.S. 1971. Brain-associated θ antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. Cell. Immunol. 2:353.

136. Dawkins, R.L., E.J. Aw and P.J. Simons. 1972. The persistence of tissue-specific antigen in muscle cells growing *in vitro*. Immunol. 23:961.

137. Dumonde, D.C. 1966. Tissue specific antigens. In F.J. Dixon and J.H. Humphrey (Eds.), Advanced in Immunology. Vol.5, Academic Press, N.Y. pp. 145-412.

138. Augusti-Tocco, G., G.H. Sato, P. Claude and D.D. Potter. 1970. Clonal cell lines of neurons. Symposia Inter. Soc. Cell Biol. Academic Press, N.Y. 9:109.

139. Muller, E. and G. Muller. 1962. Quantitative studies of the sensitivity of normal and neoplastic mouse cells to the cytotoxic action of isoantibodies. J. Exp. Med. 115:527.

140. Fish, F., I.P. Witz and G. Klein. 1974. Tumor bound immunoglobulins, the fate of immunoglobulin disappearing from the surface of coated tumor cells. Clin. Exp. Immunol. 16:355.

141. Lesley, J., R. Hyman and G. Dennert. 1974. Effect of antigen density on complement-mediated lysis, T-cell mediated killing and antigen modulation. J. Natl. Can. Inst. 53:1759.

142. Akeson, R. and H.R. Herschman. 1974. Modulation of cell-surface antigen of a murine neuroblastoma. Proc. Natl. Acad. Sci. (Wash) 71:187.

143. Zaleski, M. and J. Klein. 1977. H-2 mutation affecting immune response to Thy-1.1 antigen. J. Immunol. 145:1602.

144. Winzler, R.J. 1973. In membrane mediated information. p.1. (Kent P.W. ed.) Lancaster: MTP Medical & Technical Publ. Co.

145. Correa, M., W.J. Esselman and H.C. Miller. 1978. Thy-1 ganglioside modulation of B cell responses mimic antigenic competetion. Cellular Immunology. (Submitted for publication).

146. Ray, P.K., V.S. Thakur and K. Sundaram. 1976. Antitumor immunity. II. Viability, tumorigenicity and immunogenicity of neuraminidase-treated tumor cells: effective immunization of animals with a tumor vaccine. J. Natl. Can. Inst. 56:83.

147. Kloppel, T.M., T.W. Keenan, M.T. Freeman and D.J. Morres. 1977. Glycolipid-bound sialic acid in serum: increased levels in mice and humans bearing carcinoma. Proc. Natl. Acad. Sci. 74:3011.

148. Miller, H.C. and G. Cudkowicz. 1970. Antigen-specific cells in mouse bone marrow. I. Lasting effects of priming on immunocyte production by transferred marrow. J. Exp. Med. 132:1122.

149. Hellstrom, K.E. and I. Hellstrom. 1974. Lymphocyte-mediated cytotoxicity and blocking serum activity to tumor antigens. Adv. in Immunol. 18:209.