This is to certify that the

thesis entitled

SHEDDING OF THY-1 ALLOANTIGENS FROM T LYMPHOBLASTOID CELLS: CHARACTERIZATION OF THE RELEASED MEMBRANE COMPLEXES

presented by

WILLIAM W. FREIMUTH

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Major professor

Date 7/6/78

O-169

SHEDDING OF THY-1 ALLOANTIGENS FROM T LYMPHOBLASTOID CELLS: CHARACTERIZATION OF THE RELEASED MEMBRANE COMPLEXES

By

William W. Freimuth

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1978

ABSTRACT

SHEDDING OF THY-1 ALLOANTIGENS FROM T LYMPHOBLASTOID CELLS:

CHARACTERIZATION OF THE RELEASED MEMBRANE COMPLEXES

By

William West Freimuth

Thy-1.2 and Thy-1.1 cell surface alloantigens shed from S.49.1 (Thy-1.2, H-2^d) and BW 5147 (Thy-1.1, H-2^k) murine lymphoblastoid cells were investigated by determining the ability of culture media from these cells to induce a primary antibody response to these alloantigens. An *in vitro* modified plaque forming cell (PFC) assay with thymocytes as target cells was used to measure the formation of anti-Thy-1 antibody-producing cells. Incubation of S.49.1 culture medium with AKR/J (Thy-1.1, H-2^k) spleen cells resulted in a significant anti-Thy-1.2 PFC response against target CBA (Thy-1.2, H-2^k) thymocytes. When an equivalent amount of BW 5147 culture medium was added, no PFC responses were induced. In the reverse experiment, addition of S.49.1 or BW 5147 supernatant to CBA spleen cells resulted in only BW 5147 culture medium eliciting an anti-Thy-1.1 PFC response in a lawn of AKR/J thymocytes. Further verification of the anti-Thy-1 PFC response specificity to Thy-1 antigen resulted from congenic anti-Thy-1 sera pretreatment of immunizing culture medium which abrogated anti-Thy-1 PFC responses to the complementary Thy-1 allotype.

Rad

from S.

hours o

27.5 ho

Thy-1.2

was obs

A

line t

cultur

hemoly

anti-

activ

teste

capab

ized

sor f

togra

BW 51

shed

media

from

colum

than

of mo

ing a

Radiolabeling studies indicated two phases of shedding of Thy-1.2 from S.49.1 cells: the first occurred rapidly during the initial two hours of incubation, while the second took place between 11.5 and 27.5 hours of cultivation. After the early release of radiolabeled Thy-1.2 from S.49.1 lymphoblastoid cells, a gradual synthesis of Thy-1.2 was observed over the next ten hours of incubation.

Addition of the conditioned media from either lymphoblastoid cell line to Balb/c (Thy-1.2, H-2^d) and AKR (Thy-1.1, H-2^k) spleen cell cultures modulated approximately 50% of the primary *in vitro* anti-SRBC hemolytic plaque response. Adsorption of these two media with either anti-Thy-1.1 or anti-Thy-1.2 alloantisera abrogated the suppressive activity. However, when these same Balb/c spleen cell cultures were tested for anti-Thy-1.1 PFC responses, only BW 5147 culture medium was capable of inducing a significant PFC response which could be neutralized by anti-Thy-1.1 sera pretreatment.

Further characterization of the molecular nature of this suppressor factor, along with shed Thy-1, was carried out by column chromatography of the culture medium from glucosamine-labeled S.49.1 and BW 5147 cells. The suppressive activity was found predominantly in shed material of high molecular weight (>2 x 10^6 daltons) in conditioned media from both tumor cell lines. Thy-1.1 and Thy-1.2 antigenic activity from BW 5147 and S.49.1 cells, respectively, was detected primarily in column fractions containing substances with molecular weight greater than 2 x 10^6 daltons. In addition, pooled fractions from shed material of molecular weight estimated to be 3 x 10^5 daltons was capable of inducing anti-Thy-1 PFC responses.

Sod

molecula

culture

S.49.1 c

to dete

plexes.

molecul

shed ma

protein

be invo

labeled

lipids

togethe

Thy-1 a

complex

antiger

The fur

cell in

William West Freimuth

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of high molecular weight shed material and anti-Thy-1 immunoprecipitates of culture medium from ^{3}H-glucosamine, ^{3}H-leucine and ^{3}H-choline-labeled S.49.1 cells and ^{3}H-glucosamine-labeled BW 5147 cells was performed to determine the nature of the individual components in the shed complexes. Results from these studies indicated that the released high molecular weight complexes were heterogeneous in composition. These shed macromolecules contained significant quantities of protein, glycoprotein, glycolipid and lipid, thus suggesting a membrane fragment may be involved. Anti-Thy-1.2 precipitates of culture medium from radiolabeled S.49.1 cells consistently contained large amounts of glycolipids and a glycoprotein of approximately 53,000 daltons. Taken together, the results from all these experiments suggest that the Thy-1 antigen and modulatory factor are contained in large membrane complexes shed from these lymphoblastoid cells, and that the Thy-1 antigenic moiety may reside on both a glycolipid and a glycoprotein. The functional significance of these events in the regulation of cell-cell interactions and immune surveillance of tumor growth is discussed.

DEDICATED TO

DEBBIE

I w
Drs. Har
in my re
thank m
and Jeff
tory tec
My
Eric Eig
Arora, a
my studi
also lik
never-en
most imp
encourag

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my two advisers, Drs. Harold Miller and Walter Esselman, for their help and guidance in my research throughout my graduate program. I would also like to thank my other committee members, Drs. Tobi Jones, Ronald Patterson, and Jeffrey Williams, for their willing assistance in various laboratory techniques and helpful suggestions throughout my research project.

My sincere gratitude goes to my dear friends and colleagues, Eric Eipert, Margaret Lyerly, Linda Baum, Bill Eschenfeldt, Prince Arora, and Chris Clark, whose help, counseling, and encouragement made my studies as a graduate student an enjoyable experience. I would also like to thank Barbara Laughter and Joyce Wildenthal for their never-ending and gracious assistance in the laboratory. Finally, but most importantly, I would like to acknowledge the patience, help and encouragement of my wife, Debbie, throughout my graduate studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The structural organization and dynamic nature of the plasma membrane of eukaryotic cells and their association with a wide variety of biological functions has just begun to be appreciated. Biosynthesis and turnover of surface membrane constituents in normal and neoplastic cells have been intensively studied. However, characterization and turnover of specific cell surface components from the functional membrane have received only limited interest. Our understanding of the biological significance of these shed molecules and their role in the regulation of normal and neoplastic growth and the immune response is just evolving. The major objective of the research to be presented was to investigate the metabolism of the specific membrane component, Thy-1, found on the surface of two lymphoblastoid cells and to determine biochemical characteristics of shed complexes containing this Thy-1 antigenic moiety. This molecular complex was examined to elucidate its biological significance in relation to the regulation of antibody responses.

A review of the literature on the turnover of basic membrane constituents and the phenomenon of shedding specific cell surface components is presented. The metabolism, immunogenicity and controversy over the biochemical composition of the Thy-1 molecule expressed on the surface of thymus derived cells and certain other non-lymphoid cell types is also discussed. Finally, the role of soluble factors,

released from lymphocytes and tumor cells, in the regulation of the immune response is examined with emphasis on those substances which modulate antibody responses.

These studies were initiated from earlier *in vivo* observations in which lymphoma growth in syngeneic mice was abrogated by antiserum directed against the Thy-1 antigen on the surface of these cells. Kinetic studies of protection by this antiserum first suggested that Thy-1.2 cell surface antigen may be shed from the lymphoma cells. The remainder of this dissertation is devoted to determining the dynamics of Thy-1 release by *in vitro* studies of this Thy-1.2 tumor cell line and another, BW 5147, which bears the Thy-1.1 antigen. The turnover of Thy-1 on the surface of these lymphoblastoid cells and release into culture medium was investigated by immunoprecipitation of radiolabeled Thy-1 during various incubation periods. An *in vitro* T cell lytic plaque-forming cell assay was set up to measure immune responses directed against the Thy-1 molecule by immunizing spleen cell cultures with shed material from lymphoblastoid cells. Results of these experimental approaches are presented in the first of two manuscripts (published in J. Immunol., 120:1651). The second article (submitted for publication) focuses upon the biological function of this shed material in the modulation of antibody responses and its potential role in tumor escape from host immune surveillance. In the appendix evidence is presented to further characterize the Thy-1 moiety contained within these shed macromolecules.

Historica

degradat

tional i

branes w

and Dani

lipid bi

port of

pioneer

membran

were re

Lower c

while h

of thes

prolife

Pr

nature

membran

detail

proteins

integra

are atta

LITERATURE REVIEW

I. Shedding of Cell Surface Components

Historical Perspective. Molecular organization, biosynthesis, and degradation of plasma cell membrane components have acquired functional importance in interaction of eukaryotic cells. Plasma membranes were initially considered by Danielli in 1936 (1) and Davson and Danielli in 1952 (2) to be rigid structures consisting of a lipid bilayer surrounded by protein whose primary purpose was transport of vital nutrients and structuring the cell surface. However, pioneering investigators such as Rubin (3) suggested that the plasma membrane was dynamic in nature. He proposed that membrane components were released from cells and reinserted depending upon cell density. Lower cell densities induced the release of membrane constituents, while higher concentrations of these molecules led to reinsertion of these components, restoring membrane integrity and allowing cell proliferation.

Presently the plasma membrane is considered to be dynamic in nature as described in Singer and Nicolson's fluid mosaic model of membrane structure (4). Their model, which has been reviewed in detail (5), proposes that globular amphipathic molecules of integral proteins are partially embedded in a fluid lipid bilayer. Some integral proteins are believed to transverse the lipid bilayer and are attached or anchored to other molecules on the inner membrane

like spectrin or microfilaments (5). Edelman (6) proposes that these microfilaments are attached to microtubules allowing mobility of these integrated molecules and act as a means of intracellular communication. Another class of surface molecules are peripheral proteins, which appear to be only weakly bound to the membrane and are easily removed in aqueous buffers (4,5). The peripheral proteins are suggested to be non-covalently bound to integral proteins on the outer surface of the lipid bilayer. Carbohydrates are primarily in the form of oligosaccharide chains covalently attached to membrane bound proteins (glycoproteins) or to glycolipids (7,8). Glycolipids and gangliosides are believed to have their fatty acid tails immersed in the phospholipid bilayer with their oligosaccharide chains extending from the membrane surface into the hydrophilic periphery (8). Since the viscosity of the lipid bilayer in mammalian membranes is fluid at 37°C, this allows great mobility of most membrane constituents. The membrane fluidity permits redistribution or aggregation of surface receptors as observed in the phenomena of patching and capping (9).

Shedding: The Phenomena. Release of membrane components or fragments into the extracellular environment from functional membranes of viable cells is an event which has been described as the process of shedding (10). The origin of this phenomenon remains unclear as observations of soluble cellular or membrane components in the blood of various mammals have been described without consideration for their origin or mechanism of appearance (10). Virus budding from plasma membranes is probably the forerunner of this concept of membrane elimination. Studies of this event have revealed that insertion and removal of new membrane components is not a random process but occurs at discrete

sites on the membrane surface (11,12). Therefore, the study of shedding of membrane particles appears to be a natural process in membrane turnover or elimination.

Cell Surface Membrane Turnover *in vitro* and *in vivo*. Initial investigations on membrane turnover were performed by Warren and Glick (13), who followed the kinetics of incorporation of a variety of radioactive precursors for proteins, glycoproteins, glycolipids, and phospholipids in membranes isolated *in vitro* from cultured murine fibroblasts (L cells). Membrane composition and turnover have also been investigated by externally labeling surface components using enzymatic reactions of lactose peroxidase and galactose oxidase (14-17). Huang et al. (14) demonstrated that the rate of synthesis of membrane constituents was similar for growing and non-growing cells. Replicating cells incorporated newly synthesized components into their membrane while stationary cells eliminated membrane material at a rate equal to its synthesis (14). Warren and Glick (13) also found radiolabeled membrane material to be released into the culture medium from viable cells, suggesting an active process was involved in their shedding. There appeared to be synchronous rate of turnover between protein, carbohydrate and lipid in a random process irrespective of age (13). Metabolic inhibitors significantly slowed the rate of synthesis, followed by a concomitant decrease in the rate of degradation, suggesting a coupling of these two aspects of membrane turnover. More recent studies on the rate of synthesis and degradation of membrane macromolecules from murine L cells (18), murine lymphocytes (17), chicken embryo cells (19), murine melanoma cells (20), monkey epithelial cells (15) and neuroblastoma cells (21)

demonstrated a continuing synthesis of new membrane components to replace those lost by degradation or relase. Kaplan and Moskowitz (15) found similar rates of degradation of proteins and carbohydrates when comparing exponentially growing and contact inhibited cultures of monkey epithelial cells. In contrast to the previous results, the rate of synthesis of membrane constituents was shown to be greater in replicating cells than stationary or contact inhibited cells (15). Several researchers have shown that proteins and glycoproteins had heterogeneous rates of turnover (17-21) and sometimes were more pronounced in growing cells (15). Kaplan and Moskowitz (15) and Hubbard and Cohn (16) suggested that a significant portion of degradation was due to internalization followed by proteolysis of membrane components.

A common observation in most kinetic studies has been the biphasic rate of elimination of protein and carbohydrate surface constituents (14,15,19,20,22). A portion of the membrane components were released rapidly in the first 2-5 hours of incubation, while other components turned over at a slower rate of 2-4 days (14,15,19,20,22).

Membrane turnover studies *in vivo* have been limited but focused mainly on non-growing, long-lived liver cells (23,24) and rapidly replicating short-lived intestinal epithelial cells (25). These studies demonstrated that both cell types actively and continuously synthesize surface membranes.

Shedding and Metabolism of Specific Membrane Components

Methods. Cell surface shedding of specific membrane components has been investigated in normal lymphocytes and neoplastic cells by both biochemical and immunological approaches. Identification of membrane components that were released into their surrounding

environment

acterizatio

these compo

trypsinates

function s

labeled sh

to induce

component

Stud

normal l

a biphas

(26), H-

thymus-

nition

B-lymph

fibrobl

antiger

during

release

blasto

lympho

lowed

long i

of the

tion o

did not

medium.

surface

environment have been analyzed by 1) isolation and biochemical characterization of radiolabeled membrane components and comparison of these components to known membrane molecules or profiles of membrane trypsinates from cells under study, 2) studying of unique biological function such as enzymatic activity, 3) immunoprecipitation of radiolabeled shed material, and 4) using whole or purified shed material to induce an antibody response *in vivo* against the released membrane components.

Studies on shedding of specific membrane constituents in both normal lymphocytes and neoplastic cells *in vitro* have also suggested a biphasic release of these macromolecules. Release of Thy-1 antigen (26), H-2 antigen (18) and lectin receptors from thymocytes (27), thymus-leukemia (TL) antigen from leukemia cells (28), antigen recognition factor from murine T lymphocytes (29), cell surface Ig from B-lymphocytes (17,22,30), cell surface glycosyltransferases from fibroblasts (31), neuroblastoma cells (32), and melanoma associated antigens from murine and human tumor cells (29,33) occurs rapidly during the first six hours of incubation in fresh medium. Recently, release of Fc receptors has recently been observed from human lymphoblastoid cells (34) and Fc, SRBC, and C3 receptors from human peripheral lymphocytes (35). Kapeller et al. (19) and Bystryn (20), who followed radiolabeled membrane associated or released macromolecules for long incubation periods, have observed a slower rate of accumulation of the particular shed component. Yu and Cohen (28) studied modulation of TL by anti-TL serum on murine RADA1 leukemia cells, which did not alter the release of non-modulated TL antigens into culture medium. However, modulated TL antigens disappeared from the cell surface purportedly by endocytosis or degradation on the cell surface

(28,36). In contrast, antiserum directed against H-2 did not have any effect on the presence of H-2 antigen on the cell surface (28,36). Radiolabeled surface macromolecules of chicken embryo cells (CEC) or MAA accumulated in culture medium at a rate similar to their rate of release from the cell surface (19,20). The quantity of radiolabeled TL and MAA accumulating in the culture medium during long-term incubation was found to be greater than was originally measured on the cell surface (20,28). These results suggest that many newly synthesized membrane components are rapidly shed from the cell surface, with this process being a major metabolic mechanism of elimination of membrane components from stationary and replicating cells. The demonstration of shedding in so many different *in vitro* systems and the finding of soluble membrane components in humans and experimental animals suggests the phenomenon of shedding is a common physiological event.

Mechanism of Release of Plasma Membrane Components. Shedding has been described as a dynamic activity which is part of normal cell metabolism. Since membrane components can be found in serum or culture medium following cell death and disintegration of the cell membrane into basic components, it is necessary to demonstrate an active biosynthesis of membrane constituents and their shedding during the viable period of the cells before autolysis occurs. The initial studies of Warren and Glick (13) demonstrated metabolic inhibitors reduced membrane turnover. Cyclohexamide reduced the appearance of radiolabeled membrane components in culture medium by 50%. Cone et al. (17) determined that antimycin A or iodoacetate inhibited the release of cell surface proteins and immunoglobulins from B lymphocytes. Kappellar et al. (10,19) demonstrated that

shedding of CEC macromolecules was temperature dependent since reduction to 4°C almost completely inhibited shedding. The role of proteases in the shedding process was suggested by the observation that soybean trypsin inhibitor (100 µg/ml) and trasylol (50 units/ml) abrogated shedding by 30-50% (19). Sodium azide (10^{-5} to 10^{-2}M) had only a slightly inhibitory effect on shedding, while cyclohexamide (10 µg/ml) immediately inhibited >95% protein synthesis and in 2 hours reduced shedding by 30-50% (19). The limited studies done on the physiology of shedding suggest it is dependent upon a viable cell undergoing metabolically active membrane turnover.

Cell Cycle. A potential mechanism for regulation of shedding that has not been explored is the influence of the cell cycle. Early studies of cell cycle demonstrated that synthesis of various membrane components occurred at different times of the cell cycle (37). Glick et al. (38) demonstrated that glucosamine and choline labeled carbohydrates were incorporated primarily during the first growth phase. Studies on membrane architecture during the cell cycle have revealed that some antigens are expressed at certain stages of the cell cycle. Research by Cikes et al. (39,40) on asynchronous and synchronous murine lymphoma cells revealed that H-2 and Moloney leukemia virus-determined cell surface antigens were expressed maximally in the G_1 growth period and their expression was inversely related to the growth rate of cells. Lectin binding sites (41), HL-A antigens (42) and heparin sulfate (43) on cells have been exposed primarily during the G_1 and G_2 phase of the cell cycle. Sakiyama and Robbins (44) have recently shown that some complex glycolipids are synthesized during G_1 and early S phase. It is possible since certain antigens are

expressed maximally during the G_1 phase that shortly after their maximal expression these antigens are shed from cell surface. This event would explain the reduced quantity of antigen expressed during later stages of the cell cycle.

Composition of Shed Material. Examination of the biochemical composition of macromolecules shed from the cell surface has been limited to extracting selected membrane constituents, or a particular membrane component from whole shed material. Recent electron microscopic studies of shed material have demonstrated that membrane vesicles were released into culture medium, suggesting that these substances are liposomal in nature (45,46). Studies on the composition of shed material has demonstrated that all types of membrane constituents are present including proteins, glycoproteins, glycolipids, aminoglycans, and phospholipids (10,13-22). The exact nature of shed material containing a defined cell surface antigen or receptor as it is released from a functional cell membrane is still open to investigation.

Models of Shedding. The mechanism of shedding most commonly proposed has been clasmatosis, the pinching off of microvilli (10,26,30). Melchers et al. (22) demonstrated that different B-lymphocytes released two types of IgM at three different rates which were related to the function of the B-cells. This result could explain the biphasic release of IgM macromolecules observed in whole spleen cell population. Selectivity of release was also demonstrated in Ramsier's study (29) of T-cell receptor molecules and H-2 antigen shedding, where they observed antigen stimulation increased the release of antigen receptors but not H-2 antigens. Vitetta et al. (30) reported that some

immunoglobulins released from the cell surface of plasma cells also were attached to membrane fragments. These investigators also found that radiolabeled H-2 antigen was not released into culture medium at the same rate as surface Ig, suggesting a selectivity of release of certain components. This result was interpreted as cell surface Ig being selectively positioned in non-H-2 region on the cell surface or the shedding process selectively removing certain membrane components (30). Both of these interpretations are consistent with the selective budding of virions from plasma membranes of infected cells (11,12).

Studies on the metabolism of Thy-1 and H-2 in thymocytes also demonstrated a selective and rapid release of only Thy-1 from the cell surface (26). Considering H-2 antigens were integral proteins and suggesting Thy-1 to be a peripheral molecule, Vitteta et al. (26) postulated that either H-2 was positioned in patches in the membrane so microvilli could pinch off between H-2 antigens, or the outer layer of the lipid bilayer pinched off with only peripheral macromolecules, leaving the randomly embedded H-2 antigens on the cell surface. A recent report by Walsh and Crompton (47) demonstrated that human HLA and Ia antigens could be labeled on the inner surface of the lipid bilayer of lymphocyte plasma membrane, while human IgM and mouse IgM, IgD and Thy-1 could only be radiolabeled on the outer membrane surface. This observation suggests that cell surface Ig and Thy-1 are peripheral antigens and would agree with the suggestion of Vitetta et al. (26,30) that only peripheral macromolecules are shed.

Electron microscopic studies of shedding of murine mammary tumor virus (MuMTV) specific antigens from murine ascites leukemia cells and release of spectrin-free vesicles from human lymphocytes revealed

liposome-like structures surrounding the cell periphery (45,46). Calafat et al. (45) found that antibodies against MuMTV antigens induced redistribution of these antigens into patches and caps from which vesicles were released into culture medium containing MuMTV antigens. In contrast, Thy-1.2 and H-2.8 antigens on the cell surface were not shed in any significant quantity, again suggesting selective release of certain membrane surface components varying in each cell type. Vesicle release from "aged" human erythrocytes is dependent upon ATP depletion and not related to hemolysis (46). The "aging" process appears to be similar to shedding, as a selective release of certain membrane constituents was reported. Lutz et al. (46) observed that >95% of the macromolecules released constitute a uniform population of spheres containing major integral membrane proteins in similar quantity to that found on the cell surface except for the two-fold enrichment of acetylcholinesterase. Diverging from earlier results, the release of spectrin-free molecules represents a selective release of membrane domains lacking peripheral membrane proteins.

Biological Function of Shed Macromolecules. A wide variety of biological roles has been described or implied for membrane components shed into the external environment. Cell surface constituents released from lymphocytes have demonstrated several biological activities such as T-cell antigen receptor (29), soluble suppressor substances (48) and modulators of immune responses (49,50). Shed membrane macromolecules have been postulated to enhance tumor progression (51). Release of tumor associated antigens has been observed in several animal and human tumors (51-55). Rat sarcoma or hepatoma

tumor specific antigens (TSA) (51,54) and tumor-associated glycoprotein released from replicating TA_3-Ha tumor cells (55) enhance escape from immune destruction theoretically by blocking humoral and cellular responses, thus inhibiting direct immunological attack on tumor cells. Alexander found that rats with rapidly proliferating metastatic sarcoma tumors had a high level of soluble TSA in the tissue surrounding the tumor and in their serum (51). A concomitant reduction in immunological response against the tumor was also seen.

Shedding of membrane macromolecules has been proposed to play a role in cell replication, growth, development and differentiation. Rubin originally suggested that the release of surface components induced instability in the membrane surface, which prevented cell proliferation (3). Kapeller et al. (19) concentrated shed material from neoplastic CEC and returned it to growing CEC cells, demonstrating a significant reduction in shedding. This observation and a noticeably slower rate of shedding of cells at high densities suggested to these researchers that the quantity of shed material in the environment may affect membrane turnover of these cells (19). Aggregation-promoting factor, which enhances tissue specific association of embryonic cells to form organized tissue and developing tissue, has been isolated as a glycoprotein from the cell surface and found to accumulate in culture medium, suggesting shedding as a mechanism of release from embryonic cells (56). The colony stimulating factor of bone marrow cells is produced by a variety of cells (57). Price et al. (58) have shown the cell surface membrane to be the reservoir of colony stimulating activity. Macrophage growth factor, which is also synthesized and released into culture medium by certain cell types, has been identified as a trypsin-removable cell surface moiety (59). The

possibility exists that other soluble substances previously described in culture medium, *in vitro*, or identified in serum, *in vivo*, may have been released by the active process of shedding. Further research on the shedding phenomenon may give greater insight in such areas as membrane turnover, cellular communication, cell proliferation and regulation of the immune response.

II. Cell Surface Alloantigen Thy-1

The presence of the cell surface alloantigen Thy-1 (theta) was discovered by Reif and Allen in 1963 (60) during their investigation of a system for immune cytolysis of mouse thymic lymphocytes. Anti-Thy-1 antiserum was produced by multiple injections of AKR thymocytes intraperitoneally into C3H mice compatible in the same H-2 allele. This isoantiserum demonstrated strong cytolytic activity against thymocytes of AKR origin (60-62). Presently, the Thy-1 cell surface antigens are described as the Thy-1.1 alloantigen (formerly AKR-theta) expressed on the surfaces of AKR thymocytes and a few closely related strains (63,64). The great majority of inbred mouse strains bear the Thy-1.2 alloantigen (formerly C3H-theta) on their respective thymocyte membranes (64). Reif and Allen's expanded studies (61,62) of Thy-1 revealed that certain leukemia cells, neonatal tissue and non-lymphoid cells bear this marker, suggesting a broader scope of interest and importance as a membrane component.

Distribution of Thy-1 Antigen. Thy-1 is a widely distributed antigen found on a variety of cells and expressed in different quantities on murine lymphocytes. The Thy-1 antigen has been defined by cytotoxicity (60-62), cytolytic inhibition assays (65), and by immunofluorescent techniques (66) with either anti-thymocyte alloantiserum (60-62) or

heterologous absorbed anti-mouse brain-associated Thy-1 (BA-Thy-1) antiserum, which is reactive against both Thy-1 allotypes (67). Expression of Thy-1 antigen has been ascribed to murine thymus-derived (T) lymphocytes (60-62,68,69), lymphoblastoid cells (65), and non-thymus derived cells such as epidermal cells (70), normal and neoplastic mammary cells (71), fibroblasts in mice and rats (72) and brain tissue in mice (61,67,73), rats (74) and possibly humans (75), with the cerebral cortex containing the largest quantities in mice (73). The amount of Thy-1 antigen present on T-lymphocytes, determined by cytotoxicity (6) or immunofluorescent staining (66,77) using a fluorescent-activated cell sorter,has been used to differentiate at least two T-cell subpopulations. Thymocytes have a higher density of Thy-1 expressed on their cell surface as compared to peripheral T-lymphocytes found in the spleen or lymph nodes (61,66,69,76,77).

Thy-1 Antigen as a Differentiation Marker of Lymphocytes. Studies by Owen and Raff (68,69) on the developmental pathway of embryonic thymus stem-cell to peripheral thymus-derived lymphocytes demonstrated that Thy-1 and TL antigens were cell surface markers of differentiation. These investigators observed that Thy-1 alloantigen was not present on 14-day-old CBA embryo thymus cells. However, after 4 days in culture these embryonic cells were susceptible to cytolysis by anti-Thy-1.2 sera and complement, as were 18-day-old thymus embryo cells. These studies were confirmed *in vivo* when C3H (Thy-1.2) embryonic thymus cells were grafted into thymectomized, irradiated bone marrow-reconstituted AKR (Thy-1.1) mice. Owen and Raff observed a migration of Thy-1.2 and TL positive cells into the spleen and lymph nodes 28

days after transplantation (68). These investigators interpreted this result as an indication that a second stage of differentiation occurred. More recent studies with thymosin (78) and thymopoietin (79) have demonstrated that *in vitro* cultures of bone marrow stem cells (Thy-1 negative) can be induced to differentiate to Thy-1 positive lymphocytes upon addition of either of these thymic hormones (80,81).

Biochemical Isolation and Characterization of the Thy-1 Alloantigen. The biochemical composition of the Thy-1 antigen remains a controversy, as evidence has been presented for the protein (82,83), glycoprotein (84-97) and glycolipid (26,81,98-101) state of the Thy-1 molecule. The disagreement over the biochemical nature of Thy-1 can be partially explained by the diversity of approaches and cell types used to isolate and characterize the Thy-1 antigenic moiety. Investigators have extracted Thy-1.1 and/or Thy-1.2 from murine T-cells (26,82,92-95,98-101), brain tissue (81,95,99-101) and lymphoblastoid cells (83,96,97) and rat thymocytes and brain tissue (74,84-91). Congenic antisera, non-congenic alloantiserum and heterologous adsorbed rabbit-anti-mouse brain antiserum have been used in a variety of biochemical and immunological approaches to characterize supposedly the same or similar antigenic moiety. Therefore, in the following review of these studies one must keep in perspective the differences in methodology when examining the results.

Protein Nature of Thy-1. Atwell et al. (82) studied the nature of Thy-1 surface protein on CBA (Thy-1.2) thymocytes radiolabeled by lactoperoxidase iodination of tyrosine residues. Iodinated proteins were solubilized in a solution of 10 M urea and 1.5 M acetic

acid. The soluble and non-dialyzable cell material was immunoprecipitated in a double antibody system using anti-Thy-1.2 antiserum and excess goat anti-mouse Ig before being examined by disc gel electrophoresis on 5% sodium dodecyl sulfate (SDS) polyacrylamide gels. These experiments yielded a major radioactive peak estimated to be 60,000 molecular weight (m.w.), which they claimed was the Thy-1.2 protein (82). Kucich et al. (83) examined the presence of the Thy-1.2 molecule on the S.49.1-TB-23 lymphoblastoid cell line by inhibition of anti-Thy-1.2 cytolytic antiserum with enzyme treated S.49.1 cells. Limited digestion of S.49.1 cells with crude papain, crystalline papain, and insoluble protease prevented these cells from absorbing the anti-Thy-1.2 activity from mouse alloantisera (83). These results were interpreted as indicating that a protein moiety was necessary for Thy-1.2 activity.

Glycoprotein Nature of Thy-1

The Rat System. An antigen reactive with anti-Thy-1.1 sera has been detected on the surface of rat lymphoid and brain cells, while the Thy-1.2 allotype is not expressed in the plasma membrane of these cells (74,84-91). Acton et al. (74) estimated that there were more than 5×10^5 Thy-1.1 antigenic sites detected on 95% of rat thymocytes, 12% of spleen cells and 2% of the lymph node cells examined. Initial studies of the characterization of Thy-1.1 from rat tissue demonstrated that Thy-1.1 could be solubilized from cell membranes by a variety of non-ionic detergents and weakly ionic bile salts (84). Presence of cell surface Thy-1.1 was measured by a radioimmunoassay utilizing iodinated anti-immunoglobulins on glutaraldehye-fixed thymocytes previously treated with anti-Thy-1.1 sera in the

presence of detergent (84-88). Letarte-Muirhead et al. (84,85) demonstrated that Thy-1.1 antigenic activity could be effectively solubilized by deoxycholate and a non-ionic detergent, Lubrol-PX. Extracts were studied by gel filtration and sucrose gradients, which gave different molecular weight estimates. Gel filtration with deoxycholate columns yielded a major peak of activity between 45,000 to 65,000 m.w. Calculation of stokes radius and sedimentation values led these researchers to describe rat Thy-1.1 as a 28,000 m.w. substance (84,85). The higher molecular weight seen in column chromatography was explained by the detergent bound to the Thy-1.1 molecule (102). These researchers "purified" deoxycholate solubilized Thy-1 from rat thymocyte membranes by gel filtration and affinity chromatography on antibody or lentil lectin columns. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the Thy-1.1 molecules binding and not binding to lentil lectin yielded glycoproteins of 25,000 and 27,000 m.w., respectively, plus heterogeneous high molecular weight substances (84,85). Thy-1 purified in a similar manner from the rat brain was also a glycoprotein of about 24,000 daltons and was antigenically indistinguishable from the thymocyte Thy-1.1 antigen (86).

Rabbit antiserum prepared against rat brain was able to detect three antigens: Thy-1.1 antigen, rat-specific antigen and a cross-reacting antigen found on mouse and rat tissue by the previously described radioimmunoassay. Morris et al. (88), using identical procedures for isolating Thy-1.1, found all three antigenic moieties apparently associated with the same glycoprotein of 28,000 m.w. Studies with rat and mouse brain have also demonstrated three antigenic moieties associated with the Thy-1 found on murine

thymocytes (95). Arndt et al. (95) solubilized thymocyte brain antigen in the mouse by urea-NP-40 or deoxycholate treatment, followed by column chromatography, which showed all three Thy-1 reactive substances were 35,000 dalton substances. The serology, gel filtration and isoelectric focusing of these substances demonstrated that they were inseparable, suggesting that they were the same antigenic moiety (95).

Chemical analysis of Thy-1.1 extracted from rat thymocytes and brain indicated that the Thy-1.1 molecules were both glycoproteins of 25,000 m.w. consisting of 30% carbohydrate with similar amounts of each amino acid, but different in carbohydrate composition (89,90). Deoxycholate was removed from the Thy-1 molecule by ethanol precipitation of the purified complex. Brain Thy-1.1 has galactosamine residues and little sialic acid, while thymocyte Thy-1.1 did not have galactosamine residues (89,90). In addition, two-fold or greater differences in the quantity of fucose, galactose, glucose, and sialic acid existed between these molecules. Thymocyte Thy-1 molecules which did and did not bind to lentil lectin columns have small differences in most carbohydrate residues, which may account for differences in affinity for lectins. Thy-1 antigenicity was destroyed at 80°C for 10 minutes and by pronase, but not by other proteolytic enzymes, which suggested to these researchers that the Thy-1.1 antigenic activity resides in the protein moiety (89,90). Recently, Kuchel et al. (91) reexamined the molecular weight determinations of Thy-1 membrane glycoproteins from rat thymus and brain, to assess the influence of deoxycholate bound to these molecules after membrane solubilization. Sedimentation-equilibrium and deoxycholate-binding data demonstrated that 24% of the molecular weight was due to

deoxycholate micelles bound to the glycoproteins. In the presence of deoxycholate the thymus and brain Thy-1 glycoproteins were calculated to be 18,700 and 17,500 daltons, respectively. In the absence of deoxycholate, brain or thymus Thy-1 formed large homogeneous complexes of 270,000 or 300,000 m.w., respectively.

Murine System. Several investigators who have studied murine thymocyte surface antigen Thy-1 have concluded that it is a low molecular weight glycoprotein (92-97). Trowbridge et al. (92,94) used absorbed rabbit anti-thymocyte and anti-mouse T lymphoma sera, or anti-rat BA-Thy-1 serum to immunoprecipitate two lactose peroxidase iodinated cell surface glycoproteins of 200,000 m.w. and 25,000 m.w. and smaller quantities of other membrane material on polyacrylamide gels. The 25,000 m.w. glycoprotein was equally reactive with all three of these antisera, suggesting that this single molecule contained all three antigen specificities or that all the antigenic determinants were identical (94). Trowbridge and Hyman (93) studied several Thy-1 variants of mouse lymphoma cell lines to biochemically characterize their genetic defect. Their investigation demonstrated that the loss of the serologically defined Thy-1 antigen correlated with the absence of a 25,000 m.w. radioactive (^{3}H-mannose labeled) band corresponding to the Thy-1 antigen on the Thy-1 positive cell membrane. These investigators also established that Thy-1 was not synthesized in the Thy-1 negative variants, though some negative variants synthesized a modified Thy-1 glycoprotein containing very little carbohydrate and no galactose which was degraded rapidly (93). These authors suggested that Thy-1 antigenicity is retained in the carbohydrate moiety at the terminal sugar residues and Thy-1 negative

variants arise due to the loss of glycosyltransferases required to synthesize the precursor of Thy-1 (93).

Johnson et al. (96), using the S.49.1 TB-2.3 lymphoblastoid line, demonstrated that neuraminidase or trypsin treatment of these cells would inhibit cytolysis of the Thy-1.2-bearing S.49.1 cells by anti-Thy-1.2 sera. This result suggested that sialic acid was part of the Thy-1.2 antigenic determinant expressed upon this glycoprotein. Recently, Zwerner et al. (97) isolated and characterized the Thy-1.1 molecule from the cell surface of BW5147 lymphoblastoid cells using large-scale mammalian cell culture to produce kilogram quantities of cells. Modifications of previous procedures used in the study of rat Thy-1.1 were applied to the murine system and demonstrated that a glycoprotein of 25,000 m.w. was effective in absorbing the cytolytic activity of congenic and heterologous anti-Thy-1.1 antisera (97).

Glycolipid or Ganglioside Nature of Thy-1. Cell surface iodination of murine thymocytes and T-cells, followed by immunoprecipitation of cell lysates with congenic anti-Thy-1 sera, succeeded in isolating a Thy-1 antigenic complex (98). Vitetta et al. (98) demonstrated that the antigenicity of Thy-1 was abolished by treatment with the non-ionic detergent NP-40. Following density gradient sedimentation, virtually all of the Thy-1 antigen sedimented in the lipoprotein region of the gradient, suggesting that a lipid moiety was associated with the Thy-1 complex. Sodium dodecyl sulfate-PAGE of the iodinated Thy-1.1 and Thy-1.2 immunoprecipitates yielded a broad spectrum of radioactivity with peaks at 35,000 daltons and a high molecular weight peak near the top of the gels. Non-ionic detergent treatment of

immunoprecipitates removed radioactivity from the precipitate, but was still present in the detergent extract. Labeling of thymocytes with ^{3}H-precursors of several amino acids, fucose, and galactose demonstrated that only radiolabeled galactose was incorporated into Thy-1 antigenic complex after a four hour labeing period. Polyacrylamide gel electrophoresis of galactose labeled Thy-1 gave a broad spectrum of radioactivity from the tracking dye (migration area of glycolipids) to the 35,000 m.w. peak, suggesting a complex containing protein and glycolipid moieties (98). In another study these investigators examined the molecular weight of ^{3}H-galactose labeled Thy-1.2 released from thymocytes. The findings indicated a broad radioactive peak between 23,000 and 50,000 daltons and two small peaks of radioactivity at approximately 65,000 and 75,000 daltons, respectively (26).

Esselman and Miller (99) characterized an antigen found on both murine thymocytes and brain cells which was capable of inhibiting cytotoxicity of anti-BA-Thy-1 antiserum. Total lipid extraction from these tissues was performed by chloroform-methanol-water two-phase system in which the ganglioside rich upper phase inhibited cytotoxicity of anti-BA-Thy-1 serum (99). Thin layer chromatography was used to separate the isolated gangliosides. Only G_{D1B}, when reconstituted with cholesterol:lecithin, was completely inhibitory (99). In contrast, Arndt et al. (95) used a somewhat different method of extraction to isolate G_{D1B} from murine thymocyte membranes. Their isolated G_{D1B} was not capable of absorbing the cytotoxic activity of rabbit anti-BA-Thy-1 serum. These investigators observed that delipidation of Thy-1 antigen by organic solvents caused an 80% loss of original antigenic activity. Thy-1 antigenic activity could be

restored by lecithin and cholesterol or NP40 detergent, suggesting that a lipid moiety is essential for the antigenicity of the thymocyte-brain antigen (95). In later studies Miller and Esselman (100) found that the G_{M1} ganglioside extracted from murine thymocytes and brain could absorb the cytotoxicity of anti-Thy-1.2 sera and, to a lesser extent, rabbit anti-BA-Thy-1. Two- to four-fold greater quantities of G_{M1} from AKR mice were required to absorb anti-Thy-1.2 cytotoxicity as compared to G_{M1} from Thy-1.2-bearing C3H mice, suggesting that a specificity for the Thy-1 allotypes is found in G_{M1} ganglioside (100). Further studies on differentiating T cells demonstrated that pretreatment of bone marrow cells or thymocytes with choleratoxin or choleragenoid, which binds primarily cell surface G_{M1} (103,104), abrogated the cytotoxicity of anti-Thy-1.2 and anti-G_{M1} antisera without affecting anti-H-2 cytotoxicity (81). The neuraminidase or thymic factor treatment of bone marrow cells led to differentiation of a cell population which allowed anti-Thy-1.2 or anti-G_{M1} to lyse a significant percentage of the formerly resistant bone marrow cells. These studies suggest an unmasking or a rapid induction of expression of Thy-1.2 antigen (81). In support of these findings were the co-capping experiments of Thiele et al. (101), who used anti-Thy-1.2 and choleragen to demonstrate a common ligand-induced redistribution between these two molecules on the surface of CBA (Thy-1.2) thymocytes. However, cholera toxin did not inhibit binding of anti-Thy-1.2 as measured by immunofluorescence, suggesting to these investigators that the cholera toxin receptor is closely associated with Thy-1.2, but distinct from any of the antigenic determinants. Immunofluorescent studies by Stein-Douglas et al. (105) demonstrated that binding of anti-G_{M1} or anti-asailo G_{M1} to

murine thymocytes was not related to Thy-1 allotype, but these gangliosides were specific membrane markers for thymus-derived cells.

The exact biochemical nature of Thy-1 remains unresolved after prolonged research and often conflicting results. Presently it appears that the Thy-1 molecule contains a carbohydrate moiety and is quite complex in structure. A likely possibility to explain the variety of claims of the Thy-1 antigenic moiety is an antigenic carbohydrate moiety attached to different backbones such as protein, lipid, or sphingolipid such as is found in blood group antigens or viral antigens (106,107). If this proposal were true, it would explain the recognition of different portions of the complex Thy-1 molecule by congenic, allotypic and heterologous antisera.

Immune Response to Thy-1 Antigens. Genetic control of the anti-Thy-1 response has recently been thoroughly reviewed by Zaleski and Klein (64). The genetic locus which encodes for the cell-surface Thy-1 alloantigens is carried by chromosome 9 in mice (108). The Thy-1 locus has two alleles: one $Thy\text{-}1^{a}$ (encodes for Thy-1.1) is carried by a few closely related strains of AKR mice with an analog of this allele having been found in rats, while the $Thy\text{-}1^{b}$ allele (encodes for Thy-1.2) is carried by most wild type and inbred strains of mice that have been tested (63,64). All mouse strains examined have the ability to produce antibodies against the Thy-1 alloantigen which they lack, but the amount of antibodies produced varies with the genotype of each strain tested (64). The study of this antibody response led to the development of assays to measure both serum antibody levels and quantity of antibody forming cells *in vivo*.

Serum Antibody Test and Thy-1 Plaque Forming Cell Assay. Detection of anti-Thy-1 antibodies in the serum of mice immunized with thymocytes, bearing the other Thy-1 allotype, was tested in the presence of rabbit complement for its cytolytic ability against thymocytes of the immunizing strains (60-62,109). A more recent method of detecting and enumerating anti-Thy-1 antibody producing cells *in vivo* was devised by Fuji et al. (10) in 1970. This method is a modification of the plaque forming cell (PFC) technique described by Jerne and Nordin (111). The assay required a single intravenous injection of Thy-1.1 thymocytes into Thy-1.2 recipients (or the reverse protocol) (110). Six days later the mice were sacrificed and the number of PFC in their spleens was determined by mixing spleen cells with immunizing thymocytes in agar gel. Anti-Thy-1 antibodies diffused from antibody forming cells (embedded in agarose) into a lawn of target Thy-1 bearing thymocytes. Following incubation with rabbit complement target cells, surrounding anti-Thy-1-producing cells were lysed. Plaques were enumerated in agar gels after drying and fixing with ethanol, producing clear circular areas (plaques) against a cloudy white background.

Specificity of Anti-Thy-1 Response. Evidence that this plaque assay was actually measuring antibodies directed against the specific Thy-1 allotypes on the cell surface of target thymocytes was demonstrated by several approaches. Experiments with mice that differed in H-2 types and other cell surface membrane antigens but with identical Thy-1 allotype never showed any significant PFC response at the immunizing doses used (112-115). Plaque forming cells were detected in C3H (Thy-1.2) mice injected with AKR (Thy-1.1) thymocytes only

when thym
used as t
target ce
with C3H
cytes fro
with Thy-
compatib
were tes
could be
that onl
an anti-
of Thy-
(109,11

Ma
mary ar
when th
mice as
PFC/sp
Thy-1.
respons
elicit
PFC res
respond
respond
respons
injecti
respond

when thymocytes from any strain expressing the Thy-1.1 antigen were used as target cells, but not when Thy-1.2 bearing thymocytes were target cells (113). In the reciprocal method of injecting AKR mice with C3H thymocytes, PFC could only be detected when target thymocytes from Thy-1.2 positive strains were used. This did not occur with Thy-1.1 bearing thymocytes (114). When mice of the same histocompatibility type or congenic mice differing only in Thy-1 allotype were tested, only PFC directed against the immunizing Thy-1 allotype could be detected (115). Another indication of Thy-1 specificity was that only Thy-1 bearing tissue used for immunization would elicit an anti-Thy-1 PFC response, while tissue with the highest content of Thy-1 (thymus) gave the best results as a source of target cells (109,111,115).

Magnitude and Kinetics of Primary and Secondary Responses. Primary anti-Thy-1.1 PFC responses tested from various strains differed when they produced anti-Thy-1.1 responses. Fuji et al. (113) defined mice as high responders as $>10^4$ PFC/spleen, low responders as $<10^3$ PFC/spleen, with intermediate responders between these values. Anti-Thy-1.2 PFC responses in general were lower. Mice producing a response $>10^3$ PFC/spleen were termed high responders and mice eliciting $<10^3$ PFC/spleen were low responders (114). A measurable PFC response was first detected after 2 days for high and low responders, quickly increasing until it peaked at 4-7 days for high responders and 6 days for low responders (64,109,112). The PFC response declines rapidly in low responders such that 10 days post-injection no PFC can be detected, while it takes three weeks in high responders for the same result. Following a second thymocyte

injection, the secondary response peaks 3 days later, higher than the primary response in low responders but lower in some high responders (109). Levels of serum anti-Thy-1 antibodies correlated well with enumeration of PFC and the kinetics of responses (109). Primary responses measured PFC producing mainly IgM antibodies, while secondary responses induced IgG-producing PFC as indicated by their respective sensitivity or resistance to 2-mercaptoethanol treatment (112).

Genetic Control of the Anti-Thy-1 Response. Evidence for genetic control was first indicated by the considerable variation in anti-Thy-1 PFC response measured in several mouse strains and confirmed in studies of hybrid mice (109,116). Extensive genetic studies of the anti-Thy-1 response by Zaleski and Klein has led these investigators to propose that genetic control of the magnitude of PFC responses is carried by one major codominant gene termed Ir-Thy-1, closely linked to the H-2 complex, plus at least one minor locus (Ir-5) outside of the H-2 complex (64,116). These researchers have given a detailed hypothesis suggesting that the mechanism of gene action is similar to intermolecular antigenic competition (64).

In vitro Studies of Anti-Thy-1 Response. Anti-Thy-1.2 PFC responses were studied *in vitro* by Lake (118). He adapted Mishell and Dutton's *in vitro* culture system used to study SRBC (119) and Fuji's plaque assay (110) to measure thymocyte plaque formation. Lake used supernatant from 24 hour cultures of 6×10^7 CBA (Thy-1.2) non-stimulated thymocytes as Thy-1.2 immunizing agent for AKR (Thy-1.1) spleen cell cultures (118). Specificity of anti-Thy-1.2 response *in vitro* was demonstrated when PFC were produced only against

Thy-1.2 bearing target cells. A very low level autoantibody response was also observed when AKR thymocyte supernatant was used. Peak responses occurred on the fourth or fifth day of culture and decreased rapidly to near background three days later (118). Secondary anti-Thy-1.2 PFC responses of previously primed mice also demonstrated specificity of induction. Addition of viable CBA thymocytes to AKR spleen cell cultures would not induce an anti-Thy-1.2 PFC response, suggesting something was unique about released Thy-1 in its ability to induce *in vitro* PFC responses (118). In later work, Lake and Mitchison (120) did not find any differences in *in vitro* levels of PFC responses to Thy-1 between various mouse strains studied, in contrast to the observations by Zaleski (64).

Biological Significance of the Thy-1 Cell Surface Antigen. A biological role for the Thy-1 antigen has been postulated since Thy-1 was determined to be a differentiation antigen expressed on only those lymphocytes that were derived from the thymus or hemopoietic stem cells treated with thymic hormones. Several investigators speculated that Thy-1 antigen may play a role in the regulation of T-cell differentiation or in the immunological function of T-lymphocytes (26,68,69,80). 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 (100,121,122). Both AKR and CBA mouse brain G_{M1} gangliosides formulated into cholesterol-lecithin liposomes, suppressed anti-SRBC PFC responses when added to spleen cell cultures after one to three days of cultivation (100). Adsorption of CBA (Thy-1.2) brain G_{M1} ganglioside with anti-Thy-1.2 alloantisera would abrogate the suppressive activity of the brain

ganglioside. Preincubation of bone marrow cells or thymocytes with G_{M1} ganglioside for 24 hours before these two cell types were cultured together demonstrated that G_{M1} ganglioside only affected bone marrow cells. This result suggested that B-lymphocytes are the target of G_{M1} liposomes (100).

Culture medium from T-cell cultures (Thy-1.2), capable of suppressing antibody responses non-specifically, could be adsorbed with anti-Thy-1.2 sera or rabbit anti-G_{M1} sera to neutralize suppressive activity (121,122). These results were interpreted as indicating that the suppressive factor was Thy-1.2 or a molecule closely associated with it. Gangliosides extracted from suppressor T-cell culture medium were tested for modulatory activity. Only G_{M1} ganglioside would suppress (over 80%) SRBC responses and absorb cytotoxic activity from anti-Thy-1.2 sera (121,122). It has also been observed that within a few days following peak suppression the anti-SRBC response gradually returns to normal levels, suggesting G_{M1} glycolipid temporarily modulates the antibody response (120). These investigators have proposed that Thy-1 in a glycolipid-liposomal state is shed from antigen activated 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 normal immune response and protecting them against antigen overload or tolerance (121,122). The proposed function of Thy-1 modulating antibody responses is only one example of a variety of soluble factors released by T-cells that enhance or suppress antibody responses that will be reviewed in the next section.

III. Soluble Regulators of the Humoral Immune Response

The dichotomy of the humoral immune response into bone marrow derived (B) cells and thymus derived (T) cells was first observed in reconstitution experiments of irradiated mice. Claman (123) and Miller et al. (124) reconstituted lethally irradiated and thymectomized mice with various combinations of B cells and T cells, determining that synergy between these two cell types was required for optimal antibody production. This complex system of lymphocyte interaction has been reviewed in detail (125,126). Presently, the humoral immune response is thought to be regulated in part by at least two different subpopulations of T-lymphocytes (125,126), helper T-cells capable of amplifying antibody responses, and suppressor T-cells which regulate T helper cells resulting in reduced levels of antibody responses. These activated T-cells appear to mediate their regulatory effects upon B cells during direct cellular contact or by synthesis and release of lymphokines (125,126). Modulation of antibody responses to antigenic stimuli has also been observed in tumor-bearing animals (52,127-131). Suppression of antibody production in these hosts has been attributed to soluble factors released from proliferating neoplastic cells (51-53,127-131). Characteristics of generation, biochemical properties and mode of action of soluble regulators of the humoral response are important to the understanding of intercellular communication, control of cellular replication and manipulation of the immune response for immunotherapeutic purposes.

Amplification of Antibody Responses. A subpopulation of T-cells and soluble factors which enhance antibody responses has been studied by several researchers (125,126). Isolation of a cell-free factor from

spleen cell cultures, which could replace T-cells in *in vitro* induction of antibody responses, was first described by Rubin et al. (132), Gorczynski et al. (133) and Watson (49). These investigators showed that *in vitro* antigenic stimulation of thymocytes or purified T-cells for 24-48 hours induced the release of a soluble factor which was capable of non-specifically augmenting humoral immune responses of mouse spleen cells against SRBC antigens (49,132,133). Taussig has described an *in vivo* T-cell replacing factor which is H-2 linked and believed to be the soluble expression of the T-cell receptor (134). In addition to T-cell replacing factors, several substances such as phytohemagglutinin, concanavalin A, and pokeweed mitogen can enhance antibody responses both *in vivo* and *in vitro*, presumably by a nonclonally-restricted T-cell stimulation (125,126). Amerding et al. (135) investigated the interactions of T-cells and histoincompatible lymphocytes in mixed lymphocyte reactions. A substance termed "allogeneic effect factor" (AEF) was produced by this reaction and was capable of activating B-cells and reconstituting T-cell depleted spleen cell cultures in the development of *in vitro* antibody responses (135). Production of AEF is dependent upon H-2 differences between alloantigen active T-cells (136). Cell proliferation and DNA synthesis are not required, while protein synthesis and glycolysis are essential for AEF production (136). Biochemical characterization of AEF has determined that this substance is a glycoprotein with Ia and β_2-microglobulin determinants consisting of 40,000 dalton and 12,000 dalton subunits, both essential for its biological activity (137). T-cells which amplify antibody responses (138) and produce AEF (136) have been characterized as expressing the Ly-1^+, Ly-2, 3^- phenotype.

Soluble Suppressor Factors. Soluble factors produced by suppressor T-cells exert their modulatory effects on a variety of immunological phenomena (48). Gershon and Kondo (139) first introduced the concept of suppressor T-cells as mediators of immunological tolerance. The significance of suppressor T-cells has expanded into important roles in antigenic competition (47), mixed lymphocyte reactions (140) and regulation of immunoglobulin synthesis (48,50,141-145). Soluble effector molecules released from suppressor T-cells have been generated in cell culture by antigen-specific activation of primed lymphoid cells (50,126,141,142), non-specific mitogenic stimulation by Con A (143-145) and by specific interaction between allogeneic lymphoid cells (140). In addition, two other suppressor factors have been extracted from the plasma membrane of antigen-primed T-lymphocytes (48,146-149).

The mechanism of action and biochemical structure of soluble factors which suppress antibody responses have shown great diversity. Antigen-specific suppressive T-cell factors were extracted from carrier-primed T-cells (146) and from T-cells of nonresponder mice primed with the polymer L-glutamine^{60}-L-alamine^{30}-L-tyrosine^{10}(GAT) (147). These factors specifically suppressed the *in vitro* or *in vivo* secondary IgG PFC response against the relevant antigen only in mice syngeneic or histocompatible with the source of the suppressor factor (146,147). Taginuchi et al. (148) and Theze et al. (149) demonstrated that their suppressive factor could be absorbed by anti-Ia serum and that production of the factor was regulated by genes in the H-2 complex. The GAT suppressor factor has been characterized as a protein between 40,000-55,000 m.w. and binds directly to GAT, but its mechanism of action has yet to be determined (149). Carrier-primed

antigen specific suppressor factor (148) has been demonstrated to be a labile protein between 35,000-55,000 m.w., which affects helper T-cells by eliminating their helper activity, thus suppressing antibody responses.

Several soluble suppressor factors which were released into culture medium from antigen or mitogen-activated T-cells non-specifically suppress antibody responses to unrelated antigens (50,141-145). Thomas et al. (50) characterized soluble factors released from T-cells of ovalbumin-immune spleen cells, which suppressed anti-SRBC responses, but enhanced anti-ovalbumin responses. This suppressor factor was described as a 70°C heat stable protein of 55,000-65,000 m.w. which was active only during later stages of an antibody response. The suppressive mechanism appeared to limit antibody-producing cell proliferation rather than inhibit antibody synthesis (50). In cultures of SRBC stimulated spleen cells from mice previously immunized with horse erythrocytes (HRBC) or tetanus toxin, addition of one or both of the priming antigens elicited a soluble factor which peaked in activity between 72-120 hours of cultivation (141,142). Kempf and Rubin (141) and Douglas and Rubin (142) demonstrated that these two similar suppressor factors were both effective only when added during the first 24 hours of cultivation of spleen cells, suggesting that these factors block the initiation of antibody synthesis. Gel filtration of these factors found the suppressor activity eluting with molecules of approximately 24,000 and 34,000 daltons (141,142).

Soluble immune response suppressor (SIRS) substances produced by *in vitro* Concanavalin A-activated murine spleen cells suppresses PFC responses to SRBC *in vitro* (143,144) and *in vivo* (145). Rich et

al. (143) demonstrated that the activity of SIRS in *in vitro* spleen cell cultures was effective only when added during the initial 24 hours of cultivation, although the suppression was not manifested until the fourth or fifth day. The SIRS factor has been characterized as a 48,000-67,000 m.w. glycoprotein which also has migration inhibitory factor (MIF) activity (144). This T-cell factor (SIRS) was not cytotoxic for spleen cells and mediated suppression by acting on macrophages to suppress antibody responses. In contrast, Reinerstern et al. (145) determined that their SIRS elicited from *in vitro* Con A-stimulated spleen was less than 10,000 m.w. substance, lacked H-2 specificity and partially inhibited *in vivo* anti-SRBC PFC responses by reducing the spleen cell population. The evidence presented on the characteristics of soluble suppressor factors demonstrates that they are only a part of a complex system for the regulation of antibody response. Many mechanisms of suppression or enhancement affect B-cells, T-cells and macrophages simultaneously, indicating that the immune system is in a delicate balance receptive to the constant changes of the environment.

Suppressor Factors Released by Neoplastic Cells. Immune dysfunction in tumor-bearing animals has been repeatedly documented (51-55,127-131, 150-152), yet the mechanism of these aberrations is still not understood. Immunosuppressive soluble factors have been found in the serum and ascites fluid of many tumor-bearing animals (51-55,127-129,150-151). Tumor viruses and their products obtained from infected cells have been shown to impair a great variety of immunological functions (152). Shedding of soluble tumor specific antigens and their potential immunosuppressive effects has been proposed as a means of escaping immune

surveillance as previously described (51). Soluble factors released from a variety of neoplastic cells *in vitro* and *in vivo* have immunosuppressive activities on lymphocyte proliferation (151), frequency of rosette forming cells (127,128), graft rejection (150) and PFC responses of spleen cells (129-131).

Identification and characterization of immunosuppressive factors produced by tumor cells, which inhibit antibody synthesis, have received limited study. Kamo et al. (129) induced a marked suppression of anti-SRBC PFC response by incubation of ascites fluid or solubilized cell-free homogenates of mastocytoma cells with syngeneic spleen cells. The suppressive factor was shown to be >12,000 m.w. and heat sensitive (56°C, 30 min). Immune responsiveness could be restored by addition of SRBC-stimulated T-cells to the suppressed spleen cells *in vitro*, suggesting that the target cells were helper T-cells (153). Primary immune response of spleen cells to SRBC was inhibited by as little as 1% of culture supernatants of L1210 mouse lymphoma cells (130). Huget et al. (130) determined that the suppression was caused in part by a direct cytotoxic effect on splenic lymphocytes and macrophages by a heat labile (56°C, 30 min) and non-dialyzable substance(s). Cytolysis was not due to exhaustion of nutrients or virus contamination, as non-proliferating cells blocked by mitomycin-C still produced the cytotoxic factor as did supernatant receiving ultraviolet treatment. Addition of L1210 culture medium to spleen cells in the first two days of cultivation generated >95% suppression, as did a one hour preincubation of spleen cells with 20% culture medium, which suggested to these authors that non-proliferating T-cells were the target cells (130).

Recently, Fridman et al. (131,154) characterized a soluble factor that was spontaneously released from the ascites L-5178-Y mouse Thy-1 and Fc receptor-bearing thymoma. Earlier studies by Gisler and Fridman (155) demonstrated that *in vitro* alloantigen-activated T-cells release into culture medium immunoglobulin binding factors (IBF) which bind to the Fc fragment of IgG and block complement activation of IgG in a manner that blocks direct PFC responses to SRBC and a T-independent antigen. The lymphoma suppressive factor was shown to be identical to the IBF previously characterized (131,154). Lymphoma-IBF was most inhibitory to production of PFC responses when added three days after cultivation, similar to T-cell derived IBF, suggesting that IBF may act on the final differentiation of activated precursor cells to antibody-forming cells. Tumor-IBF and suppressor activity were removed from culture supernatants by IgG-coated sepharose columns (154). Purification and radiolabeling of the lymphoma IBF by gel filtration demonstrated that the suppressive activity eluted at 140,000 and 300,000 daltons, while SDS-PAGE of immunoprecipitates showed an 80,000 dalton substance that could be dissociated into 40,000 and 20,000 dalton subunits (154). Neauport-Sautes and Fridman (154) interpreted these results as an indication that the biologically active IBF was in a large polymeric form, while the monomeric form may represent the Fc receptor.

In summary, shedding of defined membrane components from normal and neoplastic cells is a common physiological event, often of immediate biological consequence. It is quite clear that the metabolic mechanism of shedding and the biochemical nature of the shed complexes is still evolving. Resolution of the biochemical and antigenic nature of cell surface components like Thy-1 requires new

experimental approaches and reevaluation of past research. The great diversity of soluble factors and their ever-increasing roles in regulating the immune response and cell proliferation has only started to be explored. Eventually, the mechanism of the regulation of immune responses may be understood as a complex metabolic system controlled by dynamic interactions between a variety of highly specialized and distinct cells and their soluble mediators.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Danielli, J. F. 1936. Some properties of lipoid films in relation to the structure of the plasma membrane. J. Cell Comp. Physiol. 1:393.

2. Davson, H. and J. F. Danielli. 1952. *The Permeability of Natural Membranes* 2nd ed. Cambridge Univ. Press, New York.

3. 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.

4. Singer, S. J. and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. Science 175:720.

5. Singer, S. J. 1974. Molecular biology of cellular membranes with applications to immunology. Adv. in Immunol. 19:1.

6. Edelman, G. M. 1976. Surface modulation in cell recognition and cell growth. Science 192:218.

7. Marchesi, V. T., T. W. Tillack, R. L. Jackson, J. P. Segrest, and R. E. Scott. 1972. Chemical characterization and surface orientation of the major glycoprotein of the human erythrocyte membrane. Proc. Natl. Acad. Sci. 69:1445.

8. Hakomori, S.-I. 1973. Glycolipids of tumor cell membranes. Adv. Can. Res. 18:265.

9. Schreiner, G. F. and E. R. Unanue. 1976. Membrane and cytoplasmic changes in B lymphocytes induced by ligand-surface immunoglobulin interaction. Adv. Immunol. 24:38.

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

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

12. 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.

13. 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.

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

15. Kaplan, J. and M. Moskowitz. 1975. Studies on the turnover of plasma membranes in cultured mammalian cells. I. Rate of synthesis and degradation of plasma membrane proteins and carbohydrates. Biochim. Biophys. Acta 389:290.

16. Hubbard, A. L. and Z. A. Cohn. 1975. Externally disposed plasma membrane proteins. J. Cell Biol. 64:461.

17. 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.

18. Kapeller, 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.

19. Kapeller, M., Y. M. Plesser, N. Kapeller, 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. Müller-Bérat, North-Holland Publ. Co., Amsterdam, p. 397.

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

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

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

23. Gurd, J. W. and W. H. Evans. 1973. Relative rates of degradation of mouse-liver surface-membrane proteins. Eur. J. Biochem. 36:273.

24. Kawaski, T. and I. Yamashina. 1971. Metabolic studies of rat liver plasma membranes using D-[I-^{14}C]glucosamine. Biochim. Biophys. Acta 225:234.

25. Weiser, M. M. 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. J. Biol. Chem. 248(Pt 2):2536.

26. 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.

27. Jones, G. 1973. Release of surface receptors from lymphocytes. J. Immunol. 110:1526.

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

29. Ramseier, H. 1975. Spontaneous release of T-cell receptors for alloantigens. II. Induction of antibodies to T-cell receptors. Eur. J. Immunol. 5:23.

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

31. 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.

32. 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.

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

34. Molenaar, J. L., M. van Galen, A. J. Hannema, W. Zeijlemaker, and K. W. Ponoman. 1977. Spontaneous release of Fc receptor-like material from human lymphoblastoid cell lines. Eur. J. Immunol. 7:230.

35. Sármay, G., L. István and J. Gergely. 1978. Shedding and reappearance of Fc, C3 and SRBC receptors on peripheral lymphocytes from normal donors and chronic lymphatic leukaemia (CLL) patients. Immunology 34:315.

36. Stackpole, C. W., J. B. Jacobson, and M. P. Lardis. 1974. Antigenic modulation *in vitro*. I. Fate of thymus-leukemia (TL) antigen-antibody complexes following modulation of TL antigenicity from the surfaces of mouse leukemia cells and thymocytes. J. Exp. Med. 140:939.

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

38. Glick, M. C. and C. A. Buck. 1973. Glycoproteins from the surface of metaphase cells. Biochemistry 12:85.

39. 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.

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

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

42. Everson, L. K., B. A. Plocinik, and G. N. Rogentine, Jr. 1974. HL-A expression on the G_1, S and G_2 cell-cycle stages of human lymphoid cells. J. Natl. Can. Inst. 53:913.

43. Kraemer, P. M. and R. A. Tobey. 1972. Cell-cycle dependent desquamation of heparan sulfate from the cell surface. J. Cell Biol. 55:713.

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

45. Calafat, J., J. Hilgers, W. J. Van Blitterswijk, 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.

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

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

48. Tada, T., M. Taniguchi, and T. Takemori. 1975. Properties of primed suppressor T cells and their products. Transplant. Rev. 26:106.

49. Watson, J. 1973. The role of humoral factors in the initiation of *in vitro* primary immune responses. III. Characterization of factors that replace thymus-derived cells. J. Immunol. 111:1301.

50. Thomas, D. W., W. K. Roberts and D. W. Talmage. 1975. Regulation of the immune response: production of a soluble suppressor by immune spleen cells *in vitro*. J. Immunol. 114:1616.

51. 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.

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

53. Thomson, P. M. 1975. Soluble tumor-specific antigen and its relationship to tumour growth. Int. J. Can. 15:1016.

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

55. 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.

56. 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. Nat. Acad. Sci. 70:3111.

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

58. Price, G. B., E. A. McCulloch, and J. E. Till. 1975. Cell membranes as sources of granulocyte colony stimulating activity. Expl. Haemat. 3:227.

59. Cifone, M. and V. Defendi. 1974. Cyclic expression of a growth conditioning factor (MGF) on the cell surface. Nature 252:151.

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

61. Reif, A. E. and J. M. Allen. 1966. Mouse thymic isoantigen. Nature (Lond.) 209:521.

62. 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.

63. Acton, R. T., E. P. Blankenhorn, T. C. Douglas, R. D. Owen, J. Hilgers, H. A. Hoffman and E. A. Boyse. 1973. Variations among sublines of inbred AKR mice. Nature New Biol. 245:8.

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

65. Zwerner, R. K. and R. T. Acton. 1975. Growth properties and alloantigenic expression of murine lymphoblastoid cell lines. J. Exp. Med. 142:378.

66. Cantor, H., E. Simpson, V. L. Sato, C. G. Fathman, and L. A. Herzenberg. 1975. Characterization of subpopulations of T lymphocytes. I. Separation and functional studies of peripheral T-cells binding different amounts of fluorescent anti-Thy-1.2 (theta) antibody using a fluorescence-activated cell sorter (FACS). Cell. Immunol. 15:180.

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

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

69. 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.

70. 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.

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

72. Stern, P. L. 1973. Theta alloantigen on mouse and rat fibroblasts. Nature New Biol. 246:76.

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

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

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

76. Olsson, L. and M. H. Claesson. 1973. Studies on subpopulations of theta bearing lymphoid cells. Nature New Biol. 244:750.

77. Roelants, G. E., F. Loor, H. von Boehmer, J. Sprent, L. Habb, K. S. Mayor, and A. Ryden. 1975. Five types of lymphocytes Ig-θ-, Ig-θ+ weak, Ig-θ+ strong, Ig+θ- and Ig+θ characterized by double immunofluorescence and electrophoretic mobility organ distribution in normal and nude mice. Eur. J. Immunol. 5:127.

78. Bach, J.-F. and C. Carnaud. 1976. Thymic factors. Progr. Allergy 21:342.

79. Goldstein, G., M. Scheid, V. Hammerling, E. A. Boyse, D. H. Schlesinger, and H. D. Niall. 1975. Isolation of a polypeptide that has lymphocyte differentiating properties and is probably represented universally in living cells. Proc. Natl. Acad. Sci. 72:11.

80. 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.

81. 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.

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

83. Kucich, V. N., J. C. Bennett, and B. J. Johnson. 1975. The protein nature of Thy-1.2 alloantigen as expressed by the murine lymphoblastoid line S-49.1 TB.2·3. J. Immunol. 115:626.

84. 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. 143:51.

85. 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.

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

87. 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.

88. 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.

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

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

91. Kuchel, P. W., D. G. Campbell, A. N. Barclay, and A. F. Williams. 1978. Molecular weights of the Thy-1 glycoproteins from rat thymus and brain in the presence and absence of deoxycholate. Biochem. J. 169:411.

92. 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 (Lond.) 256:652.

93. Trowbridge, I. S. and R. Hyman. 1975. Thy-1 variants of mouse lymphomas: biochemical characterization of the genetic defect. Cell 6:279.

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

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

96. 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 S-49.1 TB.2.3. J. Immunol. 116:1669.

97. Zwerner, R. K., P. A. Barstad, and R. T. Acton. 1977. Isolation and characterization of murine cell surface components. J. Exp. Med. 146:986.

98. 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.

99. Esselman, W. J. and H. C. Miller. 1974. Brain and thymus lipid inhibition of anti-brain-associated θ-cytotoxicity. J. Exp. Med. 139:445.

100. 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.

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

102. Helenius, A. and K. Simons. 1972. The binding of detergents to lipophilic and hydrophilic proteins. J. Biol. Chem. 247: 3656.

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

104. Revesz, T. and M. Greaves. 1975. Ligand-induced redistribution of lymphocyte membrane ganglioside GM1. Nature 257:103.

105. 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.

106. Pigman, W. 1977. Blood group glycoproteins. In *The Glycoconjugates*, edited by M. I. Horwitz and W. Pigman, Academic Press, New York, p. 611.

107. Burns, W. H., and A. C. Allison. 1975. Virus infections and immune responses they elicit. In *The Antigens* vol. 3, edited by M. Sela, Academic Press, New York, p. 479.

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

109. Fuji, H., M. Zaleski, and F. Milgrom. 1972. Genetic control of immune response to θ-AKR alloantigen. J. Immunol. 109:168.

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

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

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

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

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

115. 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.

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

117. Taussig, M. J. 1973. Antigenic competition. Curr. Top. Microbiol. Immunol. 60:125.

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

119. Mishell, R. I. and R. W. Dutton. 1967. Immunization of dissociated spleen cultures from normal mice. J. Exp. Med. 126: 423.

120. Lake, P. and N. A. Mitchison. 1977. Regulatory mechanisms in the immune response to cell-surface antigens. Cold Spring Harbor Symp. Quant. Biol. 41:589.

121. 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.

122. 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.

123. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow-cell combinations. Synergism in antibody production. Proc. Soc. Exp. Biol. 122:1167.

124. Miller, J. F. A. P. and G. F. Mitchell. 1968. Cell to cell interactions in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128:801.

125. Katz, P. H. and B. Benacerraf, eds. 1976. The role of products of the histocompatibility gene complex in immune responses. *Proceedings of an International Conference held at Brook Lodge, Augusta, Michigan, 1975*, Academic Press, New York, p. 485-593.

126. Gershon, R. K. 1974. T cell control of antibody production. Contemp. Top. Immunobiol. 3:1.

127. 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.

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

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

130. Huget, R. P., H.-D. Flad, and H. G. Opitz. 1977. Suppression of *in vitro* primary immune response by L1210 cells and their culture supernatant: evidence for cytotoxic effects. Cell. Immunol. 29:210.

131. Fridman, 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.

132. Rubin, A. S. and A. H. Coons. 1972. Specific heterologous enhancement of immune responses. IV. Specific generation of a thymus-derived enhancing factor. J. Exp. Med. 136:1501.

133. Gorczynski, R. M., R. G. Miller, and R. A. Phillips. 1972. Initiation of antibody production to sheep erythrocytes *in vitro*: replacement of the requirement for T-cells with a cell-free factor isolated from cultures of lymphoid cells. J. Immunol. 108:547.

134. Taussig, M. J. 1974. T cell factor which can replace T cells *in vivo*. Nature 248:234.

135. Armerding, D. and D. H. Katz. 1974. Activation of T and B lymphocytes *in vitro*. II. Biological and biochemical properties of an allogeneic effect factor (AEF) active in triggering specific B lymphocytes. J. Exp. Med. 140:19.

136. Eshhar, Z., D. Armerding, T. Waks, and D. H. Katz. 1976. Activation of T and B lymphocytes *in vitro*. V. Cellular locus, metabolism and genetics of induction and production of the allogeneic effect factor. J. Immunol. 119:1457.

137. Armerding, D., Z. Eshhar, and D. H. Katz. 1977. Activation of T and B lymphocytes *in vitro*. VI. Biochemical and physicochemical characterization of the allogeneic effect factor. J. Immunol. 119:1468.

138. Jandinski, J., H. Cantor, T. Tadakuma, D. L. Peavey, and C. W. Pierce. 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: suppressor and helper activities are inherent properties of distinct T-cell subclasses. J. Exp. Med. 143: 1390.

139. Gershon, R. K. and K. Kondo. 1970. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 18:723.

140. Rich, S. S. and R. R. Rich. 1976. Regulatory mechanisms in cell-mediated immune responses. III. I-region control of suppressor cell interaction with responder cells in mixed lymphocyte reactions. J. Exp. Med. 143:672.

141. Kempf, K. E. and A. S. Rubin. 1977. Transient suppression of the humoral immune response mediated by a factor derived from specifically activated doubly primed lymphoid cells. J. Immunol. 119:517.

142. Douglas, G. N., and A. S. Rubin. 1977. Non-specific suppression of the initiation of the immune response to a heterologous immunogen by supernatants from specifically stimulated, primed lymphoid cells. Immunology 32:669.

143. Rich, R. R. and C. W. Pierce. 1974. Biological expressions of lymphocyte activation. III. Suppression of plaque-forming cell responses *in vitro* by supernatant fluids from Concanavalin A-activated spleen cell cultures. J. Immunol. 112:1360.

144. Tanakuma, T., A. L. Kuhner, R. R. Rich, J. R. David, and C. W. Pierce. 1976. Biological expressions of lymphocyte activation. V. Characterization of a soluble immune response suppressor (SIRS) produced by Concanavalin A-activated spleen cells. J. Immunol. 117:323.

145. Reinerstern, J. L. and A. D. Steinberg. 1977. *In vivo* immune response suppression by the supernatant from Concanavalin A-activated spleen cells. J. Immunol. 119:217.

146. Takemori, T. and T. Tada. 1975. Properties of antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. I. *In vivo* activity and immunochemical characterizations. J. Exp. Med. 142:1241.

147. Kapp, J. A., C. W. Pierce, F. Delacroix, and B. Benacerraf. 1976. Immunosuppressive factor(s) extracted from lymphoid cells of non-responder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). I. Activity and antigenic specificity. J. Immunol. 116:305.

148. Taniguchi, M., K. Hayakawa, and T. Tada. 1976. Properties of antigen-specific suppressive T cell factor in the regulation of antibody response of the mouse. II. *In vitro* activity and evidence for the I region gene product. J. Immunol. 116:542.

149. Theze, J., J. A. Kapp, and B. Benacerraf. 1977. Immunosuppressive factor(s) extracted from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). III. Immunochemical properties of the GAT-specific suppressor factor. J. Exp. Med. 145:839.

150. Nowotony, A.,J. Groshman, A. Abdelnoor, N. Rote, C. Yang and R. Waltersdoff. 1974. Escape of TA3 tumors from allogeneic immune rejection: theory and experiments. Eur. J. Immunol. 4:73.

151. Gorczynski, R. M., D. G. Kilburn, 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.

152. Dent, P. B. 1972. Immunodepression by oncogenic viruses. Progr. Med. Virol. 14:1.

153. 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.

154. Neauport-Sautes, C., and W. H. Fridman. 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.

155. Gisler, R. H. and W. H. Fridman. 1975. Suppression of *in vitro* antibody synthesis by immunoglobulin-binding factor. J. Exp. Med. 142:507.

0022-1767/78/1205-1651$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 120, No. 5, May 1978
Printed in U.S.A.

RELEASE OF THY-1.2 AND THY-1.1 FROM LYMPHOBLASTOID CELLS: PARTIAL CHARACTERIZATION AND ANTIGENICITY OF SHED MATERIAL[1]

WILLIAM W. FREIMUTH,[2] WALTER J. ESSELMAN, AND HAROLD C. MILLER[3]

From the Departments of Microbiology, Public Health, and Surgery, Michigan State University, East Lansing, Michigan 48824

The ability of shed Thy-1 antigenic moiety from S.49.1 (Thy-1.2, H-2^d) and BW5147 (Thy-1.1, H-2^k) lymphoblastoid cells to induce primary antibody responses to Thy-1.1 and Thy-1.2 was investigated by using thymocytes as target cells for a plaque-forming cell (PFC) assay. Addition of S.49.1 culture medium to AKR/J (Thy-1.1, H-2^k) spleen cells induced a significant anti-Thy-1.2 PFC response against target CBA/J (Thy-1.2, H-2^k) thymocytes. In the reciprocal protocol anti-Thy-1.1 PFC responses against target AKR/J thymocytes were elicited by CBA/J spleen cells cultured with BW5147 cell culture medium. Congenic anti-Thy-1.1 sera added to immunizing culture medium provided still another test of specificity because anti-Thy-1.1 PFC responses were abrogated whereas anti-Thy-1.2 PFC responses remained unaffected. In the reverse experiment, addition of congenic anti-Thy-1.2 sera blocked the induction of anti-Thy-1.2 PFC responses.

Kinetics of Thy-1.2 release from S.49.1 cells was studied by radiolabeling the lymphoblastoid cells with ^{14}C-galactose or ^{14}C-glucosamine followed by specific immunoprecipitation of solubilized cell-associated Thy-1.2 and of shed Thy-1.2 with anti-Thy-1.2 sera. Rapid disappearance of radiolabeled Thy-1.2 from S.49.1 cells occurred during the first 2 hr of incubation followed by a gradual synthesis of Thy-1.2 over the next 10 hr. A second phase of release took place between 11.5 and 27.5 hr of incubation. Shed radiolabeled Thy-1.2 appeared rapidly in the culture medium during the first 11.5 hr phase of incubation when more than 60% of the labeled Thy-1.2 material was found to be released. Accumulation of Thy-1.2 in culture medium continued during the prolonged periods of incubation and provided increased anti-Thy-1.2 PFC responses.

The molecular properties of shed Thy-1.2 were studied by chromatography of supernatants from a 45 hr culture of ^{14}C-glucosamine labeled S.49.1 cells on a Sepharose-6B column. Thy-1.2 antigenic activity was primarily detected in fractions from a ^{14}C-radioactive peak of greater than 2 × 10^6 daltons. In addition, Thy-1 antigenic activity was found in fractions with a m.w. estimated at 3 × 10^5 daltons. These results indicate that Thy-1 is synthesized and released as a large complex from lymphoblastoid cells.

Studies on the shedding of specific alloantigens and tumor-associated antigens from normal and neoplastic cells have suggested that selective release of membrane components represents a relatively common event with important biologic function (1). Release of tumor-associated antigens has been observed in various neoplastic cells (2–7) and has been proposed as a mechanism of tumor escape from immune destruction by the host (8). Shedding of cell surface components from thymocytes and lymphocytes represent additional membrane-associated events (9–16). These membrane components have also been implicated in lymphocyte-differentiation interactions (13, 16–19).

Investigation of the nature of shed-membrane components from viable cells has focused upon the isolation and biochemical characterization of a specific biologic or immunogenic component of the membrane rather than the whole shed membrane complex from which these components may have derived. Membrane material of different compositions was found to be shed, including proteins, glycoproteins, glycosaminoglycans, and phospholipids (5, 11, 20–22). Recent electron microscopic examination of shed material from tumor cells and red blood cells has demonstrated the release of membrane vesicles resembling liposomes (4, 23).

Thy-1 antigen is a common marker of differentiation expressed on thymus-derived cells (24, 25). It is expressed in different quantities on thymocytes, mature T lymphocytes, lymphoblastoid cells (26–29) and is also found on nonthymus-derived cells such as brain tissue (30, 31), epidermal cells (32), fibroblasts (33), and mammary tumor cells (4). The biochemical nature of Thy-1 is not yet resolved, but its antigenic properties are consistent with three molecular states: protein (34), glycoprotein (35–38), and glycolipid (39, 40). Vitetta *et al.* (12) have studied the metabolism of Thy-1 in unstimulated thymocytes and demonstrated that it was rapidly released into culture medium. In our previous reports we have demonstrated a biologic function for Thy-1 and proposed that Thy-1 and G_{M1}[4] ganglioside were released from antigen-stimulated T cells

Received for publication November 4, 1977.
Accepted for publication February 10, 1978.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by Grants from the National Institutes of Health (CA-13396 and AI-12549), and the American Cancer Society (IM-158).
[2] Supported by National Institutes of Health Training Grant (GM-01911-09).
[3] Recipient of an American Cancer Society Faculty Research Award (FRA-147).

[4] Abbreviations used in this paper: G_{M1}, galactosyl-*N*-acetyl-galactosaminyl-galactosyl(*N*-acetylneuraminyl)-glucosyl-ceramide; D-MEM, Dulbecco's modified Eagle's medium containing 10% FCS; PBS, phosphate-buffered saline; NRS, normal rabbit serum; NMS, AKR/J normal mouse serum; MAA, melanoma associated antigens; TL, thymus-leukemia.

(16, 40). The shed material nonspecifically modulated antibody responses.

The antigenicity of the Thy-1 molecule on the membrane of thymocytes or in material shed from thymocytes has been studied by measuring antibody responses induced *in vivo* and *in vitro* to Thy-1 in a thymocyte plaque forming cell (PFC) assay (41–43). Lake has demonstrated that culture medium from unstimulated thymocytes incubated for 24 hr could induce *in vitro* spleen cell cultures to produce specific primary or secondary anti-Thy-1 PFC responses (43). Zaleski and Klein (44–46) investigated the genetic control of the immune response to Thy-1 by measuring the induction of anti-Thy-1 PFC responses following *in vivo* immunization with thymocytes. These investigators have demonstrated anti-Thy-1 PFC responses to be specifically induced by and directed to Thy-1 but not other alloantigens (42–45).

We now report two different experimental approaches that provide evidence for shedding of specific Thy-1 associated material by lymphoblastoid cells. The release and metabolism of Thy-1 in lymphoblastoid cells was measured by immunoprecipitation of radiolabeled Thy-1 shed into culture medium and associated with solubilized cell complexes. Shed material was also examined for antigenic activity and was capable of inducing *in vitro* antibody responses specific to Thy-1. Study of the molecular nature of this Thy-1 associated shed material suggests that it is released in the form of high m.w. complexes.

MATERIALS AND METHODS

Cells for culture and assay. Spleen cells were obtained aseptically from 10 to 16-week-old AKR (H-2^k, Thy-1.1) male mice and CBA (H-2^k, Thy-1.2) female mice (Jackson Laboratories, Bar Harbor, Maine). Cell suspensions were prepared by gentle aspiration with a syringe and needles of progressively increasing gauge (21 to 27) to obtain a single cell suspension. Spleen cells were washed once and resuspended in medium CMRL 1066 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% fetal calf serum (FCS) (Grand Island Biological Co.), 0.15 mM L-asparagine, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 mg/l gentamicin, and 2-mercaptoethanol at a final concentration of 5×10^{-5} M. Spleen cells were cultured in Marbrook culture vessels in which the cell suspension (1.0 ml) is separated from a medium reservoir (12.0 ml) by a dialysis membrane. Viability of cells was determined by trypan blue exclusion in all experiments.

Lymphoblastoid cells and supernatants. Murine lymphoblastoid cell lines S.49.1 (BALB/c, H-2^d, Thy-1.2) (29) and BW5147 (AKR/J, H-2^k, Thy-1.1) (29) were obtained from the Salk Institute Cell Distribution Center (La Jolla, Calif.). These cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Grand Island Biological Co.) with 10% heat inactivated FCS (D-MEM/FCS) supplemented with 3.5 g dextrose/l, 3.7 g $NaHCO_3$/l, and penicillin and mycostatin (each agent 100,000 units/medium) and streptomycin (100,000 μg/l). Cell-free culture supernatant containing released Thy-1 was obtained from cultures of radiolabeled and unlabeled lymphoblastoid cells usually at concentrations of 1 to 2×10^6 cells/ml by centrifugation (1600 × G) for 15 min at 4°C. Supernatants were used as Thy-1 containing immunizing agents for spleen cell cultures.

Antisera. Anti-Thy-1.2 antisera were produced in AKR/J female mice by injection of C3H or AKR/Cum thymocytes i.p. according to the method of Reif and Allen (26). Anti-Thy-1.2 antisera obtained from Litton Bionetics, Inc. (Kensington, Maryland) was used in some immunoprecipitation experiments. Anti-Thy-1.1 antisera were produced in a reverse manner by injecting AKR/J thymocytes into AKR/Cum or C3H mice. The cytotoxic titers of these pooled antisera varied with each lot and ranged from 128 to 512 when measured as previously described (40). The goat anti-rabbit Ig antisera were a gift from Dr. Ronald J. Patterson (Michigan State University).

Anti-Thy-1 plaque forming cell assay. The procedures for induction and assay of the *in vitro* primary anti-Thy-1 plaque forming cell (PFC) response are modifications of the methods of Fuji *et al.* (41) and Lake (43). Spleen cell suspensions of 2×10^7 viable cells (viability greater than 90%) were incubated with equal portions of culture medium and lymphoblastoid cell culture supernatant (final dilution 1:2 in a volume of 1.0 ml) were placed into the inner dialysis compartment of the Marbrook vessel. After the spleen cell cultures were incubated for 4 days at 37°C in a humid 8% CO_2 atmosphere, the cells from the inner chamber were aspirated and collected into pellets by centrifugation (170 × G) for 5 min at 4°C. In some experiments the aspirated cells were divided into equal parts before addition of CBA and AKR thymocytes. Thymuses were excised from 6 to 12-week-old AKR/J and CBA/J and dissected free of surrounding fascia. Thymocytes in cell suspension were washed once (300 × G) in D-MEM/FCS and resuspended in this medium. At the time of assay, viabilities and cell concentrations of cultures in each experimental group were measured. There were 2.5 to 4×10^6 viable spleen cells remaining in each group after 4 days of incubation without any discernible difference in viability or concentration when spleen cells were cultured with fresh D-MEM/FCS or lymphoblastoid cell supernatant with or without anti-Thy-1 sera. The cell pellets were resuspended in 0.1 ml of the appropriate thymocyte suspension containing 2 to 2.5×10^8 cells/ml (greater than 95% viable) in culture medium. 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, N. J.) were maintained in a 50 to 53°C water bath. The spleen-thymocyte cell suspension (20°C) was added to the heated agarose solution, vortexed, and immediately poured on a microscope slide previously dipped in a 0.1% agarose solution. After gelation, the slides were incubated upside down on specially designed slide trays and enough D-MEM/FCS was added (approximately 1.0 ml) to cover each slide. The slide trays were incubated for 4 to 4.5 hr at 37°C in a humid 8% CO_2 incubator. Each slide was drained and C (10% rabbit serum; lyophilized rabbit serum, Grand Island Biological Co., in D-MEM/FCS) was added to cover each slide for incubation of a further 45 min period. Plaques were determined by a staining technique (47) in which slides were drained and then stained with 0.2% trypan blue in 0.15 M PBS, pH 7.2, for 20 min at 20°C. After incubation, slides were rinsed twice with PBS and placed on trays and covered with PBS until the dark trypan blue stained plaques were counted under a dissecting microscope adjusted for diffuse illumination.

Radiolabeling of lymphoblastoid cells. Membrane and cell associated components of S.49.1 cells were labeled during incubation in medium containing either [1-^{14}C]-D-glucosamine-HCl or [1-^{14}C]-D-galactose (New England Nuclear, Boston, Mass.). Cells for culture were washed once, then incubated in fresh D-MEM/FCS plus radiolabeled [1-^{14}C]-D-glucosamine-HCl, 0.67 μCi/ml, 51.8 mCi/mM or [1-^{14}C]-D-galactose, 0.15 μCi/ml, 53.9 mCi/mM at a concentration of 10^6 cells/ml in a humid 8% CO_2 atmosphere at 37°C. After 24 hr the cells were washed three times in D-MEM plus 5% FCS and resuspended in culture medium at a concentration of 8×10^5 cells/ml in flasks (Falcon 3024, 75, cm^2, Oxnard, Calif.) containing 50 ml of

the radiolabeled cell suspension. Replicate culture flasks were prepared for each designated period of incubation.

Preparation of solubilized cells and incubation medium. At the end of the incubation period, cultures were removed from the incubator and viable cell counts were determined. Viabilities after each incubation period were greater than 95% and the concentration of cells had doubled during 24.5 hr of incubation. Cells were separated from culture medium by centrifugation (1600 × G) for 10 min and washed twice in PBS and then resuspended in 1.0 ml of PBS. Small aliquots of the washed cell suspension and cellfree culture medium were digested in NCS solubilizer (Amersham/Searle, Arlington Heights, Ill.) overnight at 37°C and each sample was counted in 10 ml of scintillation fluid. The remaining washed cells were lysed and solubilized by five cycles of rapid freezing and thawing and centrifuged (1600 × G) for 15 min at 4°C (39). Lysates were dialyzed against a large volume of PBS before centrifugation (10,000 × G) for 30 min at 4°C and small aliquots of supernatant were directly counted.

Immunoprecipitation of solubilized cells and cellfree culture medium. Freeze-thaw lysates (about 1.0 ml) and untreated culture medium (5.0 ml) were processed by a double antibody immunoprecipitation technique similar to Vitetta *et al.* (39) and diagrammed in Figure 1. These suspensions were first clarified by nonspecific immunoprecipitation with normal rabbit serum (NRS) and goat anti-rabbit Ig. The lysate and culture medium were then treated for 1 hr at 37°C with either 15 μl or 40 μl of AKR normal mouse serum (NMS) or anti-Thy-1.1 (controls) or anti-Thy-1.2 sera, respectively. Excess goat anti-mouse IgG (Meloy Laboratories, Springfield, Va.) was added and the mixture was incubated at 37°C for 1 hr, then overnight at 4°C, solubilized in 0.2 ml of a 5% sodium dodecyl sulfate solution, and counted as described earlier. The radioactivity in Thy-1.2 alloantigen was expressed: Thy-1.2 associated CPM = CPM anti-Thy-1.2 precipitate−CPM, anti-Thy-1.1 or AKR NMS precipitate. The ratio of CPM anti-Thy-1.2:CPM control was the following for ^{14}C-glucosamine labeled cells (1.2 to 2.1) and supernatant (5 to 10) and ^{14}C-galactose labeled cells (1.1 to 1.7) and supernatant (4 to 9). The lower ratios for the cells could be caused by greater nonspecific trapping from the more highly radiolabeled pool of solubilized cell material. Repeated immunoprecipitation of several cell and culture medium samples with anti-Thy-1.2 by the above procedure did not yield higher CPM than control precipitates, suggesting that all of the labeled Thy-1.2 material was previously precipitated.

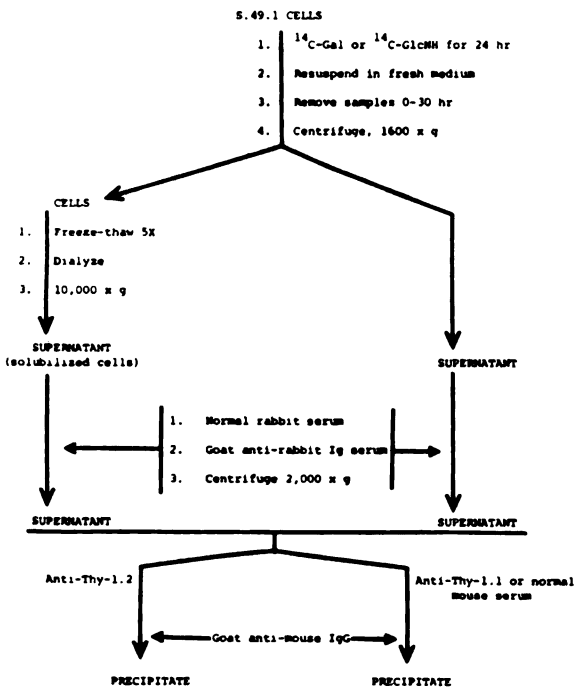

Figure 1. Protocol for preparation of and solubilization of radiolabeled S.49.1 cells and supernatants and immunoprecipitation of Thy-1. Details are provided in *Materials and Methods*.

Gel filtration of radiolabeled lymphoblastoid-cell culture supernatant. The ^{14}C-glucosamine labeled S.49.1 cellfree culture supernatants were fractionated by gel filtration over a Sepharose-6B column (Pharmacia Fine Chemicals). The Sepharose-6B column (1.5 x 60 cm) was equilibrated and run with PBS (pH 7.2) and was calibrated by using 6 m.w. markers. These included 1) blue dextran (>2 × 10^6 daltons), 2) sheep IgM (900,000 daltons), 3) sheep IgG (160,000 daltons), 4) bovine serum albumin (67,000 daltons), 5) soybean trypsin inhibitor (23,000 daltons), 6) [1-^{14}C]-D-glucosamine-HCl (216 daltons). The m.w. of identified fractions was determined as described by Reiland (48). Five to seven milliliter samples were applied to the column and 2.1 ml fractions were tested for their ability to induce a primary anti-Thy-1.2 PFC response by the usual procedure.

RESULTS

Supernatants from cultured lymphoblastoid cells were tested for the presence of Thy-1 associated complexes (Table I). Culture medium from S.49.1 (Thy-1.2) or BW5147 (Thy-1.1) cells was added to both CBA (Thy-1.2) or AKR (Thy-1.1) spleen cultures as an immunizing agent for these spleen cultures. Four to five days later they were assayed for specific anti-Thy-1 PFC responses. The only group that demonstrated a significant anti-Thy-1.1 PFC response was group 2 in which shed material from BW5147 cells, when cultured with CBA spleen cells, induced a response of 495 PFC/10^7 cells. When cells from these same groups were tested for anti-Thy-1.2 response against CBA thymocytes, only AKR spleen cells cultured with S.49.1 culture medium (group 3) demonstrated a significant response (60 PFC/10^7 cells). These data indicate that S.49.1 and BW5147 lymphoblastoid cells release Thy-1

TABLE I

Specificity of anti-Thy-1 responses induced by T-lymphoblastoid culture medium

Group	Immunizing Medium[a]	Added to Spleen Cells[b]	Anti-Thy-1.1 Response PFC/10^7 Cells (AKR Target Cells)	Anti-Thy-1.2 Response PFC/10^7 Cells (CBA Target Cells)
1	S.49.1 (1.2)[c]	CBA (1.2)	12 ± 2.5[d]	6 ± 1.6[d]
2	BW5147 (1.1)	CBA (1.2)	495 ± 72.3	9 ± 3.3
3	S.49.1 (1.2)	AKR (1.1)	12 ± 3.2	60 ± 4.9
4	BW5147 (1.1)	AKR (1.1)	5 ± 1.4	5 ± 2.4

[a] Spleen cultures with 2 × 10^7 cells in 0.5 ml medium were treated with 0.5 ml of lymphoblastoid culture medium.

[b] Cells in all groups (1 to 4) were divided equally with one-half the cells being tested against AKR (Thy-1.1) thymocytes and the other one-half tested against CBA (Thy-1.2) thymocytes.

[c] Thy-1 allotype in parentheses.

[d] Means ± standard errors of six cultures per group. This represents data from one of two similar experiments.

associated molecules into the culture medium and that this plaque assay was specific for measuring anti-Thy-1 responses.

Further verification of specificity results from adsorption of the medium with congenic anti-Thy-1 sera (Table II). Lymphoblastoid cell culture medium was incubated with anti-Thy-1.1 or anti-Thy-1.2 sera for 12 to 16 hr at 4°C. When S.49.1 culture medium was pretreated with anti-Thy-1.2, the anti-Thy-1.2 PFC response was reduced to 11 PFC/10^7 cells compared to a normal response of 192 PFC/10^7 cells. As expected, addition of anti-Thy-1.1 sera to S.49.1 culture medium did not abrogate the anti-Thy-1.2 response (220 PFC/10^7 cells). In the reverse protocol, anti-Thy-1.1 sera incubated with BW5147 medium reduced the normal anti-Thy-1.1 response of 310 PFC/10^7 cells to 6 PFC/10^7 cells. Anti-Thy-1.2 sera pretreatment of BW5147 medium had no significant effect on the anti-Thy-1.1 response.

Culture media from dividing S.49.1 cells, collected at various times of incubation, were assayed for their ability to induce anti-Thy-1.2 antibody-forming cells (Table III). After 1 hr of incubation (group 2) the medium induced a significant anti-Thy-1.2 response of 67 PFC/10^7 cells. This indicated a rapid release of shedding of Thy-1.2 soon after addition to fresh medium. Culture medium collected at 19 and 45 hr of incubation induced an increasing anti-Thy-1.2 PFC response of 138 and 167 PFC/10^7 cells, respectively. Pretreatment of culture medium from each of the previous groups with anti-Thy-1.2 sera essentially neutralized the anti-Thy-1.2 PFC response.

To determine the kinetics of release of radiolabeled Thy-1.2 associated complexes from these replicating cells, the S.49.1 lymphoblastoid cells were labeled with ^{14}C-galactose or ^{14}C-glucosamine, which are precursors of glycoproteins, glycolipids, and the Thy-1 molecule (11, 12, 20, 22, 49). Samples were collected at various time intervals and were processed according to the protocol diagrammed in Figure 1, to determine the amount of radiolabel activity associated with whole cells, solubilized cells and cellfree supernatant (Fig. 2). When precursor-labeled S.49.1 cells were placed in fresh medium, a rapid release of ^{14}C-radiolabeled material (12% of ^{14}C-glucosamine and 28% of ^{14}C-galactose total cell associated CPM) from the cells into the culture medium occurred during the first 5 hr (Fig. 2, A and B). The rate of release of radioactive material from cells prelabeled with either ^{14}C-galactose or ^{14}C-glucosamine gradually decreased between 5 and 27.5 hr of incubation such that only 13% of ^{14}C-glucosamine and 9% of ^{14}C-galactose cell associated counts were released in this 22.5 hr period.

Radiolabeled cells were frozen and thawed five times to solubilize cell membrane components (39) that were monitored for the amount of solubilized nondialyzable radioactivity. S.49.1 cells radiolabeled with either one of the carbohydrate precursors demonstrated a significant loss of radioactivity during the first five hours (Fig. 2). A slight increase and subsequent plateau of the amount of radioactivity in solubilized cell material occurred after 5 hr of incubation while there was a continual decline in the whole cell-associated radioactivity. This result suggests that radioactive precursors present inside the cell were used to biosynthesize new membrane components.

Radiolabeled Thy-1.2 found in the cell solubilized fraction and cellfree supernatant fraction was quantitated by immunoprecipitation with anti-Thy-1.2 sera according to the protocol of Figure 1. Rapid disappearance of labeled Thy-1.2 (73% of initial amount) from ^{14}C-glucosamine labeled cells took place during the first 2 hr of incubation in fresh medium (Fig. 3-A). This was followed by synthesis of Thy-1.2 (30% increase of labeled Thy-1.2) during the next 3 hr. A second phase of release of Thy-1.2 from the cell solubilized material occurred between 5 and 27.5 hr of incubation. Radiolabeled Thy-1.2 rapidly ap-

TABLE III

Kinetics of release of Thy-1.2-associated complexes into culture medium

Group No.	Immunizing Medium[a]	Hr of Incubation[b]	Anti-Thy-1.2 Response PFC/10^7 Cells (CBA Target Cells)
1	Normal		5 ± 2.1[c]
2	S.49.1	1	67 ± 18.2
3	S.49.1	19	138 ± 32.6
4	S.49.1	45	167 ± 26.9
	Medium Treated with Anti-Thy-1.2[d]		
5	Normal		2 ± 1.1
6	S.49.1	1	9 ± 3.8
7	S.49.1	19	11 ± 3.3
8	S.49.1	45	18 ± 7.9

[a] Culture medium added in the same manner as Table I to AKR (Thy-1.1) spleen cells.

[b] Culture medium collected at designated hour from cells which were washed three times and added to fresh medium at a concentration of 7.4×10^5 cells/ml; 19 hr: 10.1×10^5 cells/ml; 45 hr: 19.3×10^5/ml.

[c] Means ± standard errors of five cultures per group. This represents data from one of three similar experiments.

[d] Treatment same as Table II, except final dilution 1/160.

TABLE II

Adsorption of released Thy-1 associated complexes with Anti-Thy-1 sera

Immunizing Medium[a]	Treated with[b]	Added to Spleen Cells	Anti-Thy-1.2 Response PFC/10^7 Cells (CBA Target Cells)	Anti-Thy-1.1 Response PFC/10^7 Cells (AKR Target Cells)
1. BW5147 (1.1)[c]	—[d]	AKR (1.1)	9 ± 3.9[e]	—[f]
2. S. 49.1 (1.2)	—	AKR (1.1)	192 ± 21.2	—
3. S.49.1 (1.2)	Anti-Thy-1.2	AKR (1.1)	11 ± 5.7	—
4. S.49.1 (1.2)	Anti-Thy-1.1	AKR (1.1)	220 ± 29.6	—
5. S.49.1 (1.2)	—	CBA (1.2)	—[f]	6 ± 2.0
6. BW5147 (1.1)	—	CBA (1.2)	—	311 ± 61.2
7. BW5147 (1.1)	Anti-Thy-1.1	CBA (1.2)	—	6 ± 2.8
8. BW5147 (1.1)	Anti-Thy-1.2	CBA (1.2)	—	246 ± 27.9

[a] Culture medium added in the same manner as Table I.

[b] Culture medium was pretreated with the respective antisera (final dilution 1:40) for 12 to 16 hr at 4°C before addition to spleen cultures. S.49.1 culture medium was used from 45-hr culture shown in Table III.

[c] Thy-1 allotype in parentheses.

[d] No additions.

[e] Means ± standard errors of six cultures per group. This represents data from one of four experiments performed with congenic and allogeneic anti-Thy-1 sera, and both types of sera gave similar results.

[f] Not done. These controls are shown in Table I.

peared in the culture medium during the first 11.5 hr, when 63% of the total Thy-1.2 released in 27.5 hr was immunoprecipitated. A gradual increase in the level of labeled Thy-1.2 in the culture medium was observed during the next 16 hr of incubation.

Lymphoblastoid cells prelabeled with ^{14}C-galactose exhibited the same pattern of release as the ^{14}C-glucosamine labeled cells except that appearance of labeled Thy-1.2 in the culture medium was not as early as ^{14}C-glucosamine labeled cells (Fig. 3-B). These experiments suggest that radiolabeled Thy-1.2 was readily released into the culture medium from replicating S.49.1 lymphoblastoid cells in the first 5 to 11.5 hr of incubation. The cell associated labeled Thy-1.2 rapidly declined in the first 2 hr but was followed by a continual synthesis of new Thy-1.2, presumably from intracellular pools of radiolabeled precursors. Subsequently, this new Thy-1.2 was released causing the gradual accumulation of labeled Thy-1.2 in the culture medium during the last 16 hr of incubation.

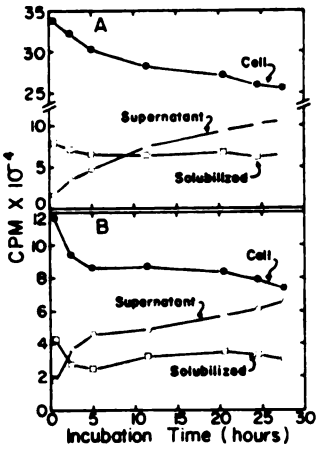

Figure 2. Fate of ^{14}C-glucosamine- (*A*) and ^{14}C-galactose- (*B*) labeled macromolecules after *in vitro* incubation of radiolabeled S.49.1 cells. Radioactivity of whole cells (●), supernatant from cell culture (○), and solubilized cells (freeze-thaw lysates) (□) was measured at intervals during the incubation period as described in Figure 1.

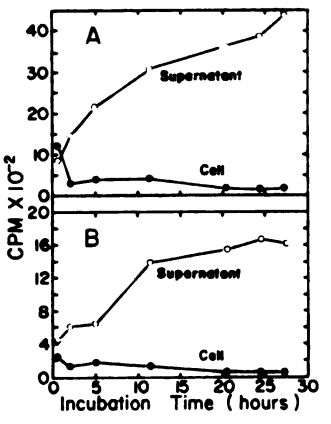

Figure 3. Fate of ^{14}C-glucosamine- (*A*) and ^{14}C-galactose- (*B*) labeled Thy-1.2 alloantigen after *in vitro* incubation of radiolabeled S.49.1 cells. Radioactivity associated with Thy-1.2 alloantigen present in supernatant from cell culture (○) and solubilized cells (freeze-thaw lysates (●) was measured at intervals during the incubation period by immunoprecipitation, as described in Figure 1.

Culture medium from a 45-hr culture of ^{14}C-glucosamine labeled S.49.1 cells was placed on a Sepharose-6B column to separate released Thy-1.2 associated complexes (Fig. 4). The major peaks of absorbance were found in fractions 31 to 40 and 42 to 49, which co-chromatographed with BSA and free amino acids, respectively. Three major peaks of radioactivity were found at fractions 15 to 19 (I), 25 to 30 (II) and 40 to 46 (III). The first contained only traces of protein and occurred at the void volume, indicating a m.w. greater than 2×10^6 daltons. Peak II had a m.w. of approximately 3×10^5 daltons and peak III contained radiolabeled metabolic products of ^{14}C-glucosamine and some protein or small peptides that were of low m.w., approximately 0.5 to 5×10^3 daltons.

Fractions that demonstrated radioactivity or absorbance were tested for their ability to induce an anti-Thy-1.2 PFC response. Only two groups of the fractions tested induced anti-Thy-1.2 PFC responses (Figure 4). Pooled fractions 16 and 17 from peak I were capable of inducing a total of 136 anti-Thy-1.2 plaques. The only other significant activity was found in pooled 27 and 28 of peak II, which induced 46 plaques. The PFC response induced by the fractions in these two peaks was nearly equal to the anti-Thy-1.2 response induced by unfractionated culture medium. Thus, practically all of the antigenic Thy-1.2 material was recovered in peaks I and II. Released Thy-1.2 associated complexes were therefore, primarily of high m.w. ($>2 \times 10^6$ daltons) with a smaller quantity of lower m.w. material ($\sim 3 \times 10^5$ daltons).

Column fractions (15 to 18, 20 to 22, 26 to 28, 32 to 34, 36 to 38 and 41 to 43) were pooled and tested for Thy-1.2 by immunoprecipitation. Peak I (15 to 18) accounted for 41% of the original precipitable counts and peak II (26 to 28) for 1%. The other fractions had no activity. The apparent loss of Thy-1.2 could be due to retention of antigen on the column or to different conditions of immunoprecipitation between crude supernatant and partially purified antigen. Comparison of percent

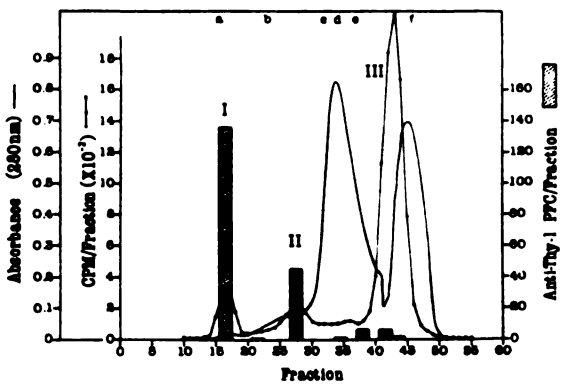

Figure 4. Sepharose-6B fractionation of supernatant from ^{14}C-glucosamine-labeled S.49.1 cells cultured for 45 hr in fresh medium. Absorbance at 280 nm (——) and cpm/fraction (●——●) were measured for each fraction. Striped bars represent an average of the total number of anti-Thy-1.2 PFC induced by the fractions tested in two to three experiments. Nonspecific control PFC were subtracted from the values presented in this figure. Molecular weight standards used to calibrate this column were: *a*, blue dextran ($>2 \times 10^6$ daltons); *b*, sheep IgM (900,000 daltons); *c*, sheep IgG (160,000 daltons); *d*, bovine serum albumin (67,000 daltons); *e*, soybean trypsin inhibitor (23,000 daltons); and *f*, [1-^{14}C]-D-glucosamine-HCl (216 daltons).

specific Thy-1.2 immunoprecipitable counts from unfractionated supernatant (about 6%) with immunoprecipitable counts from Fraction (29%) indicates a significant enrichment of Thy-1 in Fraction I.

DISCUSSION

Previous studies from our laboratory have provided evidence that complexes containing Thy-1 and glycolipid were released from antigen-stimulated T cells or suppressor cells and that these molecules were capable of modulating antibody responses (16, 40). In this report the release of Thy-1 from a homogeneous T cell population of lymphoblastoid cells was studied to determine the antigenicity and to biochemically characterize this shed material containing the Thy-1 moiety. An *in vitro* plaque forming cell assay was developed to measure antibody responses directed against released Thy-1.1 and Thy-1.2 antigenic moieties.

The observed level of *in vitro* anti-Thy-1 PFC responses induced by lymphoblastoid culture medium incubated with CBA/J and AKR/J spleen cells parallels the *in vivo* response described by Zaleski (42, 44, 45). Zaleski and Klein (46) described the immune response to Thy-1 to be under genetic control of a locus closely linked to the H-2 complex and suggested that the H-2^k locus controls anti-Thy-1.1 response. Spleen cells from CBA/J mice were found to be high responders against Thy-1.1 thymocyte target cells (44), whereas AKR/J mice were low responders against Thy-1.2-bearing thymocytes (45). The *in vivo* anti-Thy-1 PFC/spleen response of CBA mice is 10- to 20-fold greater than that of AKR/J mice (44, 45). These data can explain the 2- to 10-fold greater anti-Thy-1.1 responses compared to anti-Thy-1.2 PFC observed *in vitro*.

In the present study, significant anti-Thy-1 PFC responses were observed only when spleen cells were incubated with supernatants from lymphoblastoid cells of the Thy-1 allotype different from that on the spleen cell surface, and tested against thymocytes bearing the immunizing Thy-1 allotype (Table I). However, spleen cells incubated with culture medium from lymphoblastoid cells of the same Thy-1 allotype or tested against target thymocytes bearing this Thy-1 allotype consistently produced an apparent autoantibody response of 10 to 15% anti-Thy-1.2 PFC and only 1 to 5% of the anti-Thy-1.1 PFC. This phenomenon was also reported by Lake (43). These results could occur due to a slight cross-reactivity between the Thy-1 allotypes as observed in earlier studies (40) or represent an altered form of Thy-1 on the surface of these cells capable of inducing an autoimmune response.

Further evidence for the specificity of this response was demonstrated when congenic anti-Thy-1 sera directed against the immunizing Thy-1 allotype abrogated the specific anti-Thy-1 PFC response, whereas antisera directed against the other allotype had no effect on the PFC response (Table II). Presumably the abrogation by anti-Thy-1 sera resulted from removal of Thy-1 molecules by precipitation or by masking of the immunogenic portion of the Thy-1 molecule.

The nature of the Thy-1 molecule has led to considerable controversy over its protein (34), glycoprotein (35–38), or glycolipid (39–40) properties. Presently, there appears to be agreement that carbohydrate moieties are either antigenically associated with or part of the Thy-1 molecule. We have investigated the kinetics and nature of released material containing the Thy-1 antigenic moiety from lymphoblastoid cells by using radioactive precursors of both glycolipids and glycoproteins (^{14}C-galactose and ^{14}C-glucosamine). Recently, Van Eijk and Mulradt (49) reported that galactose and glucosamine were incorporated into murine thymocyte plasma membranes as glycoproteins and glycosphingolipids in nearly equal proportions. The general protocol that was followed for the double antibody immunoprecipitation of Thy-1 molecule from solubilized cells and culture medium was similar to that used by Vitetta *et al.* (12, 39). In the present study the immunoprecipitate, supernatant and solubilized cells were not TCA precipitated so that radiolabeled glycolipids would not be excluded from the recorded values. Rapid cycles of freezing and thawing of whole S.49.1 cells were performed for solubilization of cell and membrane components because this method nonspecifically releases surface Thy-1 molecules that retain their biochemical integrity and immunogenicity (39). Solubilization by detergent treatment can destroy the immunoprecipitability of Thy-1 (35, 39), whereas the freeze-thawing method does not seem to preclude selection of any type of molecule such as proteins or glycolipids which may be essential to Thy-1 antigenicity (34–40).

The decline of radioactivity associated with whole S.49.1 cells prelabeled with ^{14}C-galactose or ^{14}C-glucosamine paralleled a concomitant increase in radioactivity in the supernatant, suggesting that not much ^{14}C was metabolized into $^{14}CO_2$. Kappellar *et al.* (5) also found that ^{14}C-glucosamine labeled chicken embryo cells macromolecules accumulated in culture medium at a rate parallel to their rate of elimination from the cell surface. The biphasic release of radiolabeled macromolecules observed in our study was also found in studies involving turnover of membrane proteins or carbohydrates (3, 5, 14, 21, 22). The two phases consisted of a rapid release in 2 to 5 hr of incubation and a slower release of some cell surface components 2 to 4 days later (3, 5, 14, 21, 22).

The level of cell associated Thy-1.2 and other radioactive macromolecules declined rapidly during the first two to five hours of incubation, similar to that previously noted in thymocytes (12) and other studies of cell surface components (2, 3, 5, 7, 9, 14, 15). However, an increase in radiolabeled cell-associated Thy-1.2 and other macromolecules occurred during the next 10 to 15 hr of incubation, suggesting a biosynthesis of Thy-1.2 and other membrane components. An active metabolism of ^{14}C-galactose and ^{14}C-glucosamine during membrane turnover and biosynthesis of new cell products would be expected from the rapidly replicating S.49.1 lymphoblastoid cells that had doubled in the first 24 hr of incubation. Bystryn (3) observed that the amount of the radiolabeled membrane component expressed as melanoma tumor-associated antigens (MAA) initially declined and then increased 75% from the previous level during the continuous proliferation of these melanoma cells. These observations that the quantity of radiolabeled Thy-1.2 or MAA in culture medium was greater than the observed loss from cell-associated macromolecules support the contention that dividing cells synthesize more membrane components than needed to replace those lost by degradation or release (3, 5, 11, 22). These results and the fact that rapidly dividing S.49.1 cells displayed 97% viability during the entire incubation period support the proposal that the accumulation of Thy-1.2 associated material in the culture medium was an active metabolic process and not the result of debris from dying cells. In addition, these findings indicate that intracellular pools of radiolabeled precursors were present after the labeling period and were used after the initial membrane turnover of Thy-1.2 for synthesis of new Thy-1.2 membrane components. During the long term culture of S.49.1 cells a gradual reduction in the rate of appearance of radiolabeled Thy-1.2 in culture medium and in solubilized cells was noted and has been observed in similar studies on the release of MAA (2) and thymus leukemia (TL) antigens

(3). This phenomenon was probably due to a combination of higher concentrations of cells exhausting nutrients and reducing membrane biosynthesis, as well as depletion of intracellular pools of labeled precursors.

Supernatants from S.49.1 cells cultured for different periods of incubation were studied for antigenic potential in inducing an anti-Thy-1.2 PFC response (Table III). The longer the period of incubation of culture medium from S.49.1 cells, the greater was its ability to induce PFC responses, indicating an increased accumulation of Thy-1.2 in culture medium over time. Induction of anti-Thy-1.2 PFC responses was abrogated by addition of anti-Thy-1.2 sera to culture medium from these incubation periods. This result indicated that the increased presence of Thy-1.2 in culture supernatant was necessary for the increased PFC response. Other recent investigations have demonstrated that G_{M1} ganglioside with Thy-1 antigen properties was shed from antigen-stimulated T cells in sufficient quantity to modulate antibody responses (16, 40). We have also found that there is a threshold level of lymphoblastoid cell Thy-1 released into culture medium that can likewise suppress anti-Thy-1 and anti-SRBC PFC responses (unpublished results). The amount of shed Thy-1 used in the present report was below this level and any suppression that may have occurred was not apparent in these studies. Studies which suggest that shed Thy-1 has immunogenic properties and can also modulate antibody responses will be the topic of a future report.

Column chromatography of supernatant from a 45 hr culture of ^{14}C-glucosamine labeled S.49.1 cells indicated that most of the radioactivity (70%) was found in low m.w. compounds (Fig. 4, peak III). This material was not ^{14}C-glucosamine that separated on the Sepharose-6B column from the radioactive substances in peak III. These low m.w. compounds were most likely metabolic products of glucosamine (50). The fractions containing antigenic Thy-1.2 material with capacity for inducing anti-Thy-1.2 PFC responses were found only in radioactive peaks I and II. These contained substances greater than 2×10^6 daltons and approximately 3×10^5 daltons, respectively. The fractions in peak I also represented practically all the immunoprecipitable radiolabeled Thy-1.2 found in all column fractions and was enriched 5-fold in radiolabeled Thy-1.2 compared to unfractionated culture medium. The lack of antigenicity of column fractions of 27,000 to 35,000 daltons showed that the Thy-1 glycoprotein monomer (35, 38, 39) was not released.

All the Thy-1 associated counts in the supernatant or in cells were not necessarily associated with just the Thy-1 molecule, but probably were only a part of a complex containing other membrane components that also incorporated ^{14}C-galactose or ^{14}C-glucosamine. The antigenic Thy-1.2 moiety found in peak I (which contains 8% radioactivity and little protein) may be large membrane sheets or liposomes shed by the multiplying lymphoblastoid cells. Recent electron microscopic studies have revealed the release of plasma membrane vesicles from tumor cells (4) and red blood cells (23), and were previously detected in the release of cell surface immunoglobulin (9). The Thy-1.2 activity found in peak III (6% radioactivity) has a substantial amount of protein in the fractions, suggesting that the 3×10^5 daltons substances may represent aggregates of glycoprotein containing the Thy-1.2 antigenic moiety, or a breakdown product of the high m.w. substances. Further biochemical characterization of these Thy-1 active peaks is presently under investigation.

In conclusion, these studies on the metabolism of Thy-1 in lymphoblastoid cells during long term culture have demonstrated that these membrane associated molecules were rapidly released into culture medium before any detectable replication of cells. During further cell incubation Thy-1 was actively biosynthesized and subsequently released. These molecules in Thy-1.1 or Thy-1.2 alloantigen states were capable of inducing specific PFC response directed against the immunizing Thy-1 allotype on the surface of target thymocytes. The radiolabeled Thy-1 was released predominantly as a high m.w. macromolecule. Functional significance of these events is presently being investigated to determine the role of Thy-1 regulation of cell-cell interactions.

REFERENCES

1. Doljanski, F., and M. Kapeller. 1976. Cell surface shedding—the phenomenon and its possible significance. J. Ther. Biol. 62:253.
2. Yu, A., and E. P. Cohen. 1974. Studies on the effect of specific antisera on the metabolism of cellular antigens. II. The synthesis and degradation of TL antigens of mouse cells in the presence of TL antiserum. J. Immunol. 112:1296.
3. Bystryn, J. C. 1976. Release of tumor-associated antigens by murine melanoma cells. J. Immunol. 116:1302.
4. Calafat, J., J. Hilgers, W. J. Van Blitterswijk, M. Verbett, 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. Cancer Inst. 56:1019.
5. Kapeller, M., Y. M. Plesser, N. Kapeller, 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 Publishing Co., Amsterdam. P. 397.
6. Molenaar, J. L., M. vanGalen, A. J. Hannema, W. Zeijlemaker, and K. W. Ponoman. 1977. Spontaneous release of Fc receptor-like material from human lymphoblastoid cell lines. Eur. J. Immunol. 7:230.
7. Bystryn, J.-C. 1977. Release of cell-surface tumor-associated antigens by viable melanoma cells from humans. J. Natl. Cancer Inst. 59:325.
8. 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.
9. Vitetta, E. A., and J. W. Uhr. 1972. Cell surface immunoglobulin. V. Release from murine splenic lymphocytes. J. Exp. Med. 136:676.
10. Jones, G. 1973. Release of surface receptors from lymphocytes. J. Immunol. 110:1526.
11. Kapeller, 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.
12. 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.
13. Gisler, R. H., and W. H. Fridman. 1975. Suppression of *in vitro* antibody synthesis by immunoglobulin-binding factor. J. Exp. Med. 142:507.
14. Melchers, F., R. E. Cone, H. vonBoehmer, and J. Sprent. 1975. Immunoglobulin turnover in B lymphocyte subpopulations. Eur. J. Immunol. 5:382.
15. Ramseier, H. 1975. Spontaneous release of T cell receptors for alloantigens. II. Induction of antibodies to T cell receptors. Eur. J. Immunol. 5:23.
16. 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.
17. Watson, J. 1973. The role of humoral factors in the initiation of *in vitro* primary immune responses. III. Characterization of factors that replace thymus-derived cells. J. Immunol. 111:1301.
18. Thomas, D. W., W. K. Roberts, and D. W. Talmage. 1975. Regulation of the immune response: Production of a soluble suppressor by immune spleen cells *in vitro*. J. Immunol. 114:1616.

19. Tadakuma, T., A. L. Kuhner, R. R. Rich, J. R. David, and C. W. Pierce. 1976. Biological expressions of lymphocyte activation. V. Characterization of a soluble immune response suppressor (SIRS) produced by concanavalin A-activated spleen cells. J. Immunol. 117:323.
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. Huang, C. C., C.-M. Tsai, and E. S. Canellakis. 1973. Iodination of cell membranes. II. Characterization of HeLa cell membrane surface proteins. Biochim. Biophys. Acta 332:59.
22. Kaplan, J., and M. Moskowitz. 1975. Studies on the turnover of plasma membranes in cultured mammalian cells. I. Rate of synthesis and degradation of plasma membrane proteins and carbohydrates. Biochim. Biophys. Acta 389:290.
23. Lutz, H. U., S.-C. Liv, and J. Palek. 1977. Release of spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of spectrin-free vesicles. J. Cell. Biol. 73:548.
24. Scheid, M. P., G. Goldstein, J. Hammerling, and E. A. Boyse. 1975. Lymphocyte differentiation from precursor cells *in vitro*. Ann. N. Y. Acad. Sci. 249:531.
25. 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.
26. Reif, A. E., and J. M. Allen. 1966. Mouse thymic isoantigen. Nature 209:521.
27. Cantor, H., E. Simpson, V. L. Sato, C. G. Fathman, and L. A. Herzenberg. 1975. Characterization of subpopulations of T lymphocytes. I. Separation and functional studies of peripheral T-cells binding different amounts of fluorescent anti-Thy-1.2(theta) antibody using a fluorescence-activated cell sorter (FACS). Cell. Immunol. 15:180.
28. Roelants, G. E., F. Loor, H. vonBoehmer, J. Sprent, L. Hagg, K. S. Mayor, and A. Ryden. 1975. Five types of lymphocytes Ig-θ-, Ig-θ+ weak, Ig-θ+ strong, Ig+θ- and Ig+θ characterized by double immunofluorescence and electrophoretic mobility organ distribution in normal and nude mice. Eur. J. Immunol. 5:127.
29. Zwerner, R. K., and R. T. Acton. 1975. Growth properties and alloantigenic expression of murine lymphoblastoid cell lines. J. Exp. Med. 142:378.
30. 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.
31. Golub, E. S. 1971. Brain-associated theta antigen: Reactivity of rabbit anti-mouse brain with mouse lymphoid cells. Cell Immunol. 2:353.
32. 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.
33. Stern, P. L. 1973. Theta alloantigen on mouse and rat fibroblasts. Nature (New Biol.) 246:76.
34. Kucich, U. N., J. C. Bennett, and B. J. Johnson. 1975. The protein nature of Thy-1.2 alloantigen as expressed by the murine lymphoblastoid line S-49.1 TB.2.3. J. Immunol. 115:626.
35. 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.
36. 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.
37. 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 S-49.1 TB.2.3. J. Immunol. 116:1669.
38. Williams, A. F., A. N. Barclay, M. Letarte-Muirhead, and A. J. Morris. 1977. Rat Thy-1 antigens from thymus and brain: Their tissue distribution, purification, and chemical composition. *In* Cold Spring Harbor Symp. Quant. Biol. 41:51.
39. 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.
40. 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.
41. Fuji, H., R. T. Schultz, and F. Milgrom. 1970. Cytolysis in agar of thymus cells by antibody-forming cells. Proc. Soc. Exp. Biol. Med. 133:180.
42. Fuji, H., M. Zaleski, and F. Milgrom. 1971. Immune response to alloantigens of thymus studied in mice with plaque assay. J. Immunol. 106:56.
43. Lake, P. 1976. Antibody response induced *in vitro* to the cell-surface alloantigen Thy-1. Nature 262:297.
44. Zaleski, M. B. 1974. Immune response of mice to Thy-1.1 antigen: Studies on congenic lines. Immunogenetics 1:226.
45. Zaleski, M. B. 1975. Preliminary evidence of genetic control of the immune response to the Thy-1.2 antigen in mice. Immunogenetics 2:21.
46. Zaleski, M., and J. Klein. 1977. H-2 mutation affecting immune response to Thy-1.1 antigen. J. Immunol. 145:1602.
47. Taylor, G. M., and J. Bennett. 1973. A modified plaque test for the detection of cells forming antibody to alloantigens. J. Immunol. Meth. 2:213.
48. Reiland, J. 1971. Gel filtration. Methods Enzymol. 22:287.
49. Van Eijk, R. V. W., and P. F. Mühlradt. 1977. Carbohydrate incorporation in plasma membranes of mouse thymocytes stimulated by concanavalin A. Eur. J. Biochem. 78:41.
50. Plagemann, P. G. W., and J. Erbe. 1973. Transport and metabolism of glucosamine by cultured Novikoff rat hepatoma cells and effects on nucleotide pools. Cancer Res. 33:482.

Depar

SOLUBLE FRACTORS CONTAINING THY-1 ANTIGEN SHED FROM LYMPHOBLASTOID CELLS MODULATE *IN VITRO* PLAQUE FORMING CELL RESPONSE[1]

W. W. Freimuth, H. C. Miller and W. J. Esselman[2]

Departments of Microbiology and Public Health and of Surgery

Michigan State University

East Lansing, Michigan 48824

1. Th
of
So
2. W.
Gra
Can
3. Abbr
acety
ceram
Dulbe
phosph
mouse
Thy-1;
electro

FOOTNOTES

1. This work was supported by grants from the National Institutes of Health (CA-13396 and AI-12549), and the American Cancer Society (IM-158).

2. W.W.F. is supported by National Institutes of Health Training Grant (GM-01911-09), and H.C.M. is the recipient of an American Cancer Society Faculty Research Award (FRA-147).

3. Abbreviations: PFC, plaque-forming cells; G_{M1}, galactosyl-N-acetyl-galactosaminyl-galactosyl (N-acetylneuraminyl)-glucosyl-ceramide; D-MEM, Dulbecco's modified Eagle's medium; D-MEM/FCS, Dulbecco's Modified Eagle's medium containing 10% FCS; PBS, phosphate-buffered saline; NRS, normal rabbit serum; NMS, normal mouse serum; BSA, bovine serum albumin; BA-Thy-1, brain associated Thy-1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ABSTRACT

Culture media from two lymphoblastoid cell lines BW 5147 (Thy-1.1, H-2^k) and S.49.1 (Thy-1.2, H-2^d) were capable of suppressing approximately 50% of the primary *in vitro* anti-SRBC hemolytic plaque response of Balb/c (Thy-1.2, H-2^d) and AKR (Thy-1.1, H-2^k) spleen cell cultures. The association of the Thy-1 molecule with the suppressor factor was suggested when pretreatment of both of these conditioned media with either anti-Thy-1.1 or anti-Thy-1.2 alloantisera abrogated the modulatory activity. Simultaneous measurement of primary anti-Thy-1.1 PFC responses by the same Balb/c (Thy-1.2, H-2^d) spleen cell cultures indicated that only BW 5147 culture medium could induce a significant anti-Thy-1.1 antibody response. In contrast to neutralization of suppressor activity by both anti-Thy-1 alloantisera, only anti-Thy-1.1 sera adsorption of BW 5147 culture medium abrogated the anti-Thy-1.1 PFC response, while anti-Thy-1.2 sera enhanced it. These results suggested a dissociation between the antigenic Thy-1 moiety and the suppressive activity detected in lymphoblastoid culture medium.

The molecular properties of the suppressor factor and shed Thy-1 were analyzed by column chromatography of culture medium from ^{3}H-glucosamine radiolabeled S.49.1 and BW 5147 cells. The suppressive activity in culture medium from both tumor cells was found in fractions of high molecular weight (>2 x 10^6 daltons). Immunoprecipitation techniques and the anti-Thy-1 PFC assay were utilized to demonstrate that in both neoplastic cell lines the Thy-1 antigenicity also resided primarily in large molecular weight complexes of >2 x 10^6 daltons. Column fractions containing high molecular weight substances

from BW 5147 or S.49.1 culture medium, either by themselves or pretreated with anti-Thy-1.1 or anti-Thy-1.2 sera were cultivated with Balb/c spleen cell cultures and gave results similar to those observed with unfractionated culture medium. Pretreatment of either lymphoblastoid culture medium with normal mouse serum did not affect this suppressive activity in anti-SRBC PFC responses nor their ability to induce specific anti-Thy-1 PFC responses. These experiments indicate that the suppressor activity found in the conditioned medium of these two lymphoblastoid cells is contained in a high molecular weight complex that may be associated with the Thy-1 antigen.

INTRODUCTION

Cell surface components released into their surrounding media by normal and neoplastic cells have been implicated as soluble regulators of a variety of immune responses (1-4). Soluble effector molecules extracted or shed from the membranes of antigen or mitogen activated T-cells are capable of suppressing or enhancing antibody responses (5-14). The mechanisms of suppression of antibody responses by soluble T-cell derived factors are both antigen-specific and non-specific in nature (5-9,15,16). The generation of soluble suppressor factors and their inhibitory mechanisms of action are often encoded by genes of the major histocompatibility complex (2,6-8,15). Modulation of antibody responses has also been observed in tumor-bearing animals (4,16-18) and *in vitro*, when spleen cell cultures were incubated with ascites fluid (18,19) or culture supernatant of neoplastic cells (20-22). It has been postulated that membrane macromolecules shed from proliferating tumor cells enhance tumor progression by providing protection against immune destruction by the host (3).

The shedding of membrane glycolipids, glycoproteins, lipids and proteins has been proposed as a common mechanism of elimination for cell surface constituents during the rapid rate of membrane turnover observed in various cell types (1,23-27). Our understanding of the exact nature of shed material and its relation to defined cell surface antigens or receptors, during release from functional cell membranes, is still evolving. Shed material from tumor cells and red blood cells which have been examined by electron microscopy reveal the presence of membrane vesicles which resemble artificial liposomes (28,29).

We have previously described a modulatory substance that was released from antigen-stimulated T-cells, which temporarily blocks the terminal differentiation of B-cells into plasma cells (30,31). The modulatory activity was found to be associated with a glycolipid isolated from suppressor culture medium that had properties consistent with G_{M1} ganglioside. Neutralization of this suppressor activity by anti-Thy-1 and anti-G_{M1} sera indicated that Thy-1 antigen and G_{M1} ganglioside were associated with the shed modulatory T-cell products.

Vitetta et al. (32) initially observed that Thy-1 antigen was selectively released from unstimulated thymocytes, while H-2 antigens remained on the cell surface. Recently, we reported that Thy-1.1 and Thy-1.2 alloantigens were shed from BW 5147 and S.49.1 lymphoblastoid cells, respectively (33). Release of Thy-1 antigen was determined by immunoprecipitation techniques and the use of an *in vitro* spleen cell immune response assay (33-36). This assay measures anti-Thy-1 PFC responses specifically induced by and directed to Thy-1 but not other cell surface alloantigens (33-36). Evidence is

now presented that select components of culture medium from these lymphoblastoid cells modulate *in vitro* antibody responses. The association of Thy-1 antigen with this suppressor activity is discussed.

MATERIALS AND METHODS

Cells for culture and assay. Spleen cells were obtained aseptically from 10 to 16 week old AKR ($H\text{-}2^k$, Thy-1.1) male mice and 12-24 week old Balb/c ($H\text{-}2^d$, Thy-1.2) female mice (Jackson Laboratories, Bar Harbor, ME). Cell suspensions were prepared by gentle aspiration with a syringe and needles of progressively increasing gauge (21-27) to obtain a single cell suspension. Spleen cells were washed once and resuspended in medium CMRL 1066 (Grand Island Biological Co.), 0.15 mM L-aspargine, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 mg/l gentamicin, and 2-mercaptoethanol at a final concentration of 5×10^{-5}M. Spleen cells were cultured in Marbrook culture vessels in which the cell suspension (1.0 ml) is separated from a medium reservoir (12.0 ml) by a dialysis membrane (33).

Thymuses used as source of target cells in anti-Thy-1 plaque forming cell assay were excised from 10 to 16 week old AKR/J mice and dissected free of surrounding fascia. Single cell suspensions were obtained by mincing the thymuses with forceps followed by successive aspiration with syringe and needles. Cells were washed once (300 x g) and then resuspended in Dulbecco's MEM with 10% FCS medium (33). Thumus cells obtained this way have viability greater than 95% by trypan blue exclusion method.

Lymphoblastoid cells and supernatants. Murine lymphoblastoid cell lines S.49.1 (Balb/c, $H\text{-}2^d$, Thy-1.2) and BW 5147 (AKR/J, $H\text{-}2^k$,

Thy-1.1) were obtained from the Salk Institute Cell Distribution Center (La Jolla, CA). These cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Grand Island Biological Co.) with 10% heat inactivated FCS (D-MEM/FCS) supplemented with 3.5 g dextrose/l, 3.7 g $NaHCO_3$/l, and penicillin and mycostatin (each agent 100,000 units/l) and streptomycin (100,000 µg/l). Cell free culture supernatant containing released Thy-1 was obtained from cultures of radiolabeled and unlabeled lymphoblastoid cells at concentrations of 1-2 x 10^6 cells/ml by centrifugation (1,600 x g) for 15 minutes at 4°C (33). Supernatants were used as Thy-1 containing immunizing agents for spleen cell cultures and as a source of suppressor factors. Lymphoblastoid culture medium was concentrated three to five fold on CF-50A centriflo membrane cones (Amicon Corp., Lexington, MA) to obtain concentrated fractions of material >50,000 m.w. following column chromatography.

Antisera. Anti-Thy-1.2 antisera were produced in AKR/J female mice by injection of C3H or AKR/Cum thymocytes intraperitoneally according to the method of Reif and Allen (37). Anti-Thy-1.2 antisera obtained from Litton Bionetics, Inc. (Kensington, MD) was used in some immunoprecipitation experiments. Anti-Thy-1.1 antisera were produced in a reverse manner by injecting AKR/J thymocytes into AKR/Cum or C3H mice. The cytotoxic titers of these pooled antisera varied with each lot and ranged from 128 to 512 when measured as previously described (30). The goat anti-rabbit Ig antisera were a gift from Dr. Ronald J. Patterson (Michigan State University). A final dilution of 1:40 was used to adsorb out Thy-1 antigenicity and suppressive activity in lymphoblastoid culture medium for 12-16 hours at 4°C before addition to spleen cell cultures (33).

Antigen. Sheep erythrocytes (SRBC) were obtained from a single animal (Grand Island Biological Co., Grand Island, NY) and were stored in Alsever solution. Before use, the SRBC were washed three times in sterile phosphate buffered saline (PBS) and suspended to 1 x 10^9 cells/ml in spleen cell culture medium.

Hemolytic plaque-forming cell (PFC) assay. Spleen cell suspensions of 2 x 10^7 viable cells (viabilities greater than 85%) in 0.2 ml of spleen cell culture medium were added to 0.8 ml of lymphoblastoid culture supernatant and 0.05 ml of SRBC which were placed into the inner dialysis compartment of the Marbrook vessel. Pooled column fractions from concentrated lymphoblastoid culture medium (0.5 ml representing ∿1.0 ml of unfractionated medium) were added to spleen cells in 0.5 ml of 2X spleen cell culture medium plus SRBC. Fractions 35-38 and 40-43 or effluent of <50,000 m.w. substances, that passed through membrane cones in a volume of 0.8 ml, was added to Marbrook chambers in the same manner as whole culture medium. To control for the effects of increased concentrations of normal components found in culture medium following column chromatography, normal unconditioned medium (D-MEM/FCS) was also fractionated in an identical manner as lymphoblastoid culture medium and added to spleen cell cultures in identical quantities. Normal unfractionated D-MEM/FCS (control) was added to spleen cell cultures in identical quantity (0.8 ml) as whole lymphoblastoid culture medium. After the spleen cell cultures were incubated for five days at 37°C in a humid 8% CO_2 atmosphere, the cells from the inner chamber were aspirated and collected. At the time of assay, viabilities and cell concentrations in each experimental group were measured. There were an average of 2.0-2.5 x 10^6 viable

spleen cells remaining in each group after five days of cultivation. No discernible difference in viability or concentration was detected when spleen cells were cultured with fresh D-MEM/FCS of lymphoblastoid supernatant with or without anti-Thy-1 sera. Suspensions from these spleen cell cultures (0.1 ml) were assayed by the Jerne hemolytic plaque method as modified for use with agarose gel on glass microscope slides. Details of this procedure are described elsewhere (38).

$$\% \text{ Suppression} = \frac{\text{PFC (Lymphoblastoid supernatant or pooled fractions)}}{\text{PFC (Normal Medium or Control Fractions)}} \times 100$$

Anti-Thy-1 plaque forming cell assay. The procedures for induction and assay of the *in vitro* primary anti-Thy-1 plaque forming cell (PFC) response have been previously described in detail (33). The spleen cells remaining after the assay for anti-SRBC PFC response were collected into pellets by centrifugation (170 x g) for 5 minutes at 4°C. The cell pellets were resuspended in 0.1 ml of the AKR/J thymocyte suspension containing 2-2.5 x 10^8 cells/ml (greater than 95% viable) in culture medium. 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 50-53°C water bath. The spleen-thymocyte cell suspension (20°C) was added to the heated agarose solution, vortexed, and immediately poured on a microscope slide previously dipped in a 0.1% agarose solution. After gelation, the slides were incubated upside down on specially designed slide trays and enough D-MEM/FCS was added (approximately 1.0 ml) to cover each slide. The slide trays were incubated for 4 to 4.5 hours at 37°C in a humid

8% CO_2 incubator. Each slide was drained and complement (10% rabbit serum; lyophilized rabbit serum, Grand Island Biological Co., in D-MEM/FCS) was added to cover each slide for incubation of a further 45 minute period. Plaques were determined by a staining technique (39) in which slides were drained and then stained with 0.2% trypan blue in 0.15 M phosphate buffered saline, pH 7.2 (PBS) for 20 minutes at 20°C. Following incubation, slides were rinsed twice with PBS and placed on trays and covered with PBS until the dark trypan blue stained plaques were counted under a dissecting microscope adjusted for diffuse illumination.

Radiolabeling of lymphoblastoid cells. Membrane and cell associated components of S.49.1 and BW 5147 cells were labeled during incubation in medium containing [6-^{3}H(N)]-D-glucosamine-HCl (New England Nuclear, Boston, MA). Cells for culture were washed once, then incubated in fresh D-MEM/FCS with only 1000 mg/l glucose plus radiolabel [6-^{3}H(N)]-D-glucosamine-HCl, 2.0 μCi/ml, 29.0 Ci/mmole at a concentration of 10^6 cells/ml in a humid 8% CO_2 atmosphere at 37°C. After 24 hours the cells were washed three times in D-MEM plus 5% FCS and resuspended in complete culture medium at a concentration 8 x 10^5 cells/ml in roller bottles containing 250 ml of the radiolabeled cell suspension. At the end of the 40 hour incubation period, cultures were removed from the incubator and viable cell counts were determined. Viabilities were greater than 95% and the concentration of cells had doubled during 24.5 hours of incubation. Culture medium was separated from cells by centrifugation (160 x g) for 10 minutes.

Immunoprecipitation of cell-free culture medium and column fractions. Radiolabeled lymphoblastoid culture medium (5.0 ml) or

pooled column fractions (4.0 ml) were processed by a double antibody immunoprecipitation technique described previously (33). These suspensions were first clarified by non-specific immunoprecipitation with normal rabbit serum (NRS) and goat anti-rabbit Ig. The culture medium and column fractions were then treated for 1 hour at 37°C with 10 µl of anti-Thy-1.1 or anti-Thy-1.2 sera for each milliliter of sample. Excess goat anti-mouse IgG (Meloy Lab) was added and the mixture was incubated at 37°C for 1 hour then overnight at 4°C, solubilized in 0.2 ml of a 5% sodium dodecyl sulfate solution, and counted in 10 ml of scintillation fluid. The radioactivity in Thy-1.2 alloantigen was expressed: Thy-1.2 associated CPM = CPM anti-Thy-1.2 precipitate - CPM anti-Thy-1.1. Thy-1.1 associated radioactivity (CPM) = CPM anti-Thy-1.1 precipitate-CPM anti-Thy-1.2 precipitate.

$$\% \text{ Thy-1 immunoprecipitated} = \frac{\text{Pooled fractions Thy-1 assoc. CPM}}{\text{Total fractions Thy-1 assoc. CPM}} \times 100$$

Repeated immunoprecipitation of several culture medium samples with anti-Thy-1.2 or anti-Thy-1.1 by the above procedure did not yield higher CPM than control precipitates, suggesting that all of the labeled Thy-1 material was previously precipitated.

Gel filtration of radiolabeled lymphoblastoid-cell culture supernatant. The ^{3}H-glucosamine labeled S.49.1 and BW 5147 cell-free culture supernatants were fractionated by gel filtration over a Sepharose-6B column (Pharmacia Fine Chemicals, Piscataway, NJ). The Sepharose-6B column (1.5 x 60 cm) was equilibrated and run with PBS (pH 7.2) and was calibrated using the following molecular weight markers: 1) blue dextran (>2 x 10^{6} daltons), 2) sheep IgM (900,000 daltons), 3) sheep IgG (160,000 daltons), 4) bovine serum albumin (67,000 daltons), 5) soybean trypsin inhibitor (23,000 daltons) and

6) [6-^{3}H(N)]-D-glucosamine-HCl (216 daltons). The molecular weight of identified fractions was determined as described by Reiland (40). Seven milliliter samples of untreated or concentrated culture medium were applied to the column and pooled 2.1 ml fractions were tested for: their ability to induce a primary anti-Thy-1 PFC response, immunoprecipitation with anti-Thy-1 sera, and ability to suppress hemolytic PFC responses.

RESULTS

Culture medium from S.49.1 (Thy-1.2, H-2^{d}) and BW 5147 (Thy-1.1, K-2^{k}) T-lymphoblastoid cells were tested for their ability to suppress primary *in vitro* anti-SRBC PFC responses of AKR/J (Thy-1.1, H-2^{k}) and Balb/c (Thy-1.2, H-2^{d}) spleen cells (Table I). Cultivation of AKR/J spleen cells with either S.49.1 or BW 5147 culture medium for five days in Marbrook chambers with SRBC as test antigen reduced the control mean PFC response (4547) in culture to 2610 and 2072 PFC/culture, respectively. S.49.1 and BW 5147 lymphoblastoid supernatant suppressed the normal Balb/c spleen cell PFC response (2875) to a similar degree of approximately 50% to 1417 PFC/culture and 1355 PFC/culture. Lymphoblastoid culture medium had no effect on either Balb/c or AKR/J spleen cell viabilities during the five day cultivation period.

Since we have previously demonstrated that Thy-1 was shed from these tumor cells (33) and that this released material from antigen-activated suppressor T-cells could modulate *in vitro* antibody responses (30,31), we decided to test the lymphoblastoid culture medium for the presence of a modulatory effect associated with Thy-1 (Table II). S.49.1 and BW 5147 culture media were incubated with anti-Thy-1 sera for 12-16 hours at 4°C before addition to Balb/c spleen cell cultures.

TABLE I

MODULATION OF ANTI-SRBC RESPONSE BY T-LYMPHOBLASTOID CULTURE MEDIUM

SPLEEN CELLS[a]	CULTURE MEDIUM	ANTI-SRBC RESPONSE PFC/CULTURE
AKR/J (1.1)	---[b]	4547 ± 245[d]
AKR/J	S.49.1 (1.2)[c]	2610 ± 270
AKR/J	BW 5147 (1.1)	2072 ± 234
BALB/c (1.2)	---	2875 ± 244
BALB/c	S.49.1 (1.2)	1417 ± 149
BALB/c	BW 5147 (1.1)	1355 ± 97

[a]Spleen cell cultures with 2 x 10^7 cells in 0.2 ml medium were treated with 0.8 ml of lymphoblastoid culture medium and 0.05 ml of a 1.5% SRBC solution in spleen cell culture medium.

[b]No addition of lymphoblastoid culture medium.

[c]Thy-1 allotype in parentheses.

[d]Means ± standard errors of six cultures per group.

TABLE II

ADSORPTION OF MODULATORY EFFECT BY ANTI-THY-1 SERA

	CULTURE[a] MEDIUM	TREATED WITH[c]	ANTI-SRBC RESPONSE PFC/CULTURE	ANTI-THY-1.1 RESPONSE PFC/10^7 CELLS (AKR TARGET CELLS)
1.	NORMAL	---[d]	1617 ± 144[e]	---[f]
2.	S.49.1 (1.2)[b]	---	540 ± 90	14 ± 8.3[e]
3.	BW 5147 (1.1)	---	641 ± 81	196 ± 34.1
4.	NORMAL	Anti-Thy-1.2	1519 ± 73	---
5.	S.49.1 (1.2)	Anti-Thy-1.2	1218 ± 179	---
6.	S.49.1 (1.2)	Anti-Thy-1.1	1871 ± 269	---
7.	BW 5147 (1.1)	Anti-Thy-1.2	1392 ± 153	409 ± 35.2
8.	BW 5147 (1.1)	Anti-Thy-1.1	1706 ± 256	4 ± 1.1
9.	BW 5147 (1.1)	NO SRBC	79 ± 8	198 ± 36.6

[a]BALB/c spleen cell cultures with 2 x 10^7 cells in 0.2 ml medium were treated with 0.8 ml of lymphoblastoid culture medium and 0.05 ml of 1.5% SRBC in culture per group. Each culture was simultaneously tested for anti-SRBC and anti-Thy-1.1 PFC responses.

[b]Thy-1 allotype in parentheses.

[c]Culture medium was pretreated with the respective antisera (final dilution 1:40) for 12-16 hours at 4°C before addition to spleen cell cultures.

[d]No additions.

[e]Means ± standard errors of six cultures per group.

[f]NOT DONE (These controls have been done previously, with no induction of PFC).

Addition of anti-Thy-1.2 (fourth group) or anti-Thy-1.1 sera (not shown) to normal (unconditioned) culture medium had no significant effect on the mean normal (group I) PFC response (1617 vs 1519 PFC/culture) or on cell viability. Pretreatment of S.49.1 supernatant with either anti-Thy-1.2 or anti-Thy-1.1 sera abrogated the suppressive effects of this culture medium (540 PFC/culture, group 2), returning the PFC responses to 1218 and 1871 PFC/culture (groups 5 and 6, respectively). Adsorption of BW 5147 culture medium with anti-Thy-1.2 and anti-Thy-1.1 sera also removed the inhibitory activity allowing the induction of a normal anti-SRBC PFC response (groups 7 and 8, respectively). Addition of AKR/J or C3H normal mouse serum to lymphoblastoid culture medium had no effect on the modulatory activity (not shown). Taken together these results suggest that conditioned medium from lymphoblastoid cultures contains a Thy-1 associated suppressor complex.

To demonstrate the presence of the Thy-1.1 antigenic moiety in lymphoblastoid culture medium and the specificity of anti-Thy-1 alloantiserum, the *in vitro* anti-Thy-1 plaque forming cell assay was used (33-36). Specificity of this assay for the Thy-1 allotype present in immunizing culture medium and on the surface of Thy-1 bearing thymocytes (target cells) has been described (33-36). The same spleen cells assayed for anti-SRBC PFC responses were tested for anti-Thy-1.1 PFC responses. Thy-1 specificity was demonstrated when only BW 5147 culture medium (group 3) and not S.49.1 culture medium (group 2) could induce a significant anti-Thy-1.1 PFC response (196 vs 14 PFC/10^7 cells). Pretreatment of BW 5147 culture medium with anti-Thy-1.1 sera (group 8) completely abrogated the normal anti-Thy-1.1 PFC response

of 196 PFC/10^7 cells to 4 PFC/10^7 cells. Anti-Thy-1.2 sera, on the other hand, did not prevent the induction of Thy-1.1 PFC responses but, in fact, in this experiment significantly enhanced the response to 409 PFC/10^7 (group 7). To determine if the presence of SRBC affected the anti-Thy-1.1 response, a control group was set up in which SRBC were not added to cultures containing BW 5147 supernatant (group 9). The lack of SRBC did not affect anti-Thy-1.1 PFC response. The specificity of anti-Thy-1.1 PFC responses, coupled with observation of anti-Thy-1 sera directed against either Thy-1 allotype neutralizes the suppressive activity in BW 5147 or S.49.1 medium, suggests that the Thy-1 antigenic moiety is separate but closely associated with the suppressive factor.

Culture medium from 40 hour cultures of ^{3}H-glucosamine labeled BW 5147 and S.49.1 cells were fractionated on a Sepharose-6B column to separate released Thy-1 associated complexes and suppressor factors of antibody responses (Figure 1). Selected column fractions (15-18, 20-23,25-28,30-33,35-38,40-43) were pooled and tested for the presence of radiolabeled Thy-1 by immunoprecipitation with anti-Thy-1.1 and anti-Thy-1.2 alloantisera (Figure 1-A). Pooled fractions 15-18 from BW 5147 lymphoblastoid culture medium accounted for 94.0% of anti-Thy-1.1 immunoprecipitable counts found in all the fractions tested. Similarly, fractions 15-18 of S.49.1 supernatant contained 95.2% of all the radiolabeled Thy-1.2 precipitable counts as previously observed (33). Fractions 20-23, and 25-28 from both cell types contained very low levels (2-3%) of precipitable counts. A ten-fold enrichment of Thy-1 immunoprecipitable counts was found in fractions 15-18 compared to those found in whole medium. Counts in these higher

Figure 1. Sepharose-6B fractionation of supernatants from ^{3}H-glucosamine-labeled S.49.1 and BW 5147 cells cultured for 40 hours in fresh medium. A. Results from anti-Thy-1 immunoprecipitation of radiolabeled Thy-1.1 or Thy-1.2 in BW 5147 and S.49.1 culture medium, respectively. B. Suppression of anti-SRBC PFC responses by pooled fractions of lymphoblastoid supernatant. Percent suppression represents: the amount of modulation of normal PFC response (culture containing Sepharose-6B fractions of unconditioned culture medium) compared to cultures containing identical fractions of either BW 5147 or S.49.1 supernatant. Data presented are pooled from three experiments. C. Absorbance at 280 nm (———) and CPM/fraction (●——●——●) were measured for each fraction. Striped bars represent an average of the total number of anti-Thy-1 PFC induced by the fractions tested in 2 to 3 experiments. Non-specific control PFC were subtracted from the values presented in this figure. Molecular weight standards used to calibrate this column were: (a) blue dextran (>2 x 10^{6} daltons), (b) sheep IgM (900,000 daltons), (c) sheep IgG (160,000 daltons), (d) bovine serum albumin (67,000 daltons), (e) soybean trypsin inhibitor (23,000 daltons), and (f) [1-^{14}C]-D-glucosamine-HCl (216 daltons).

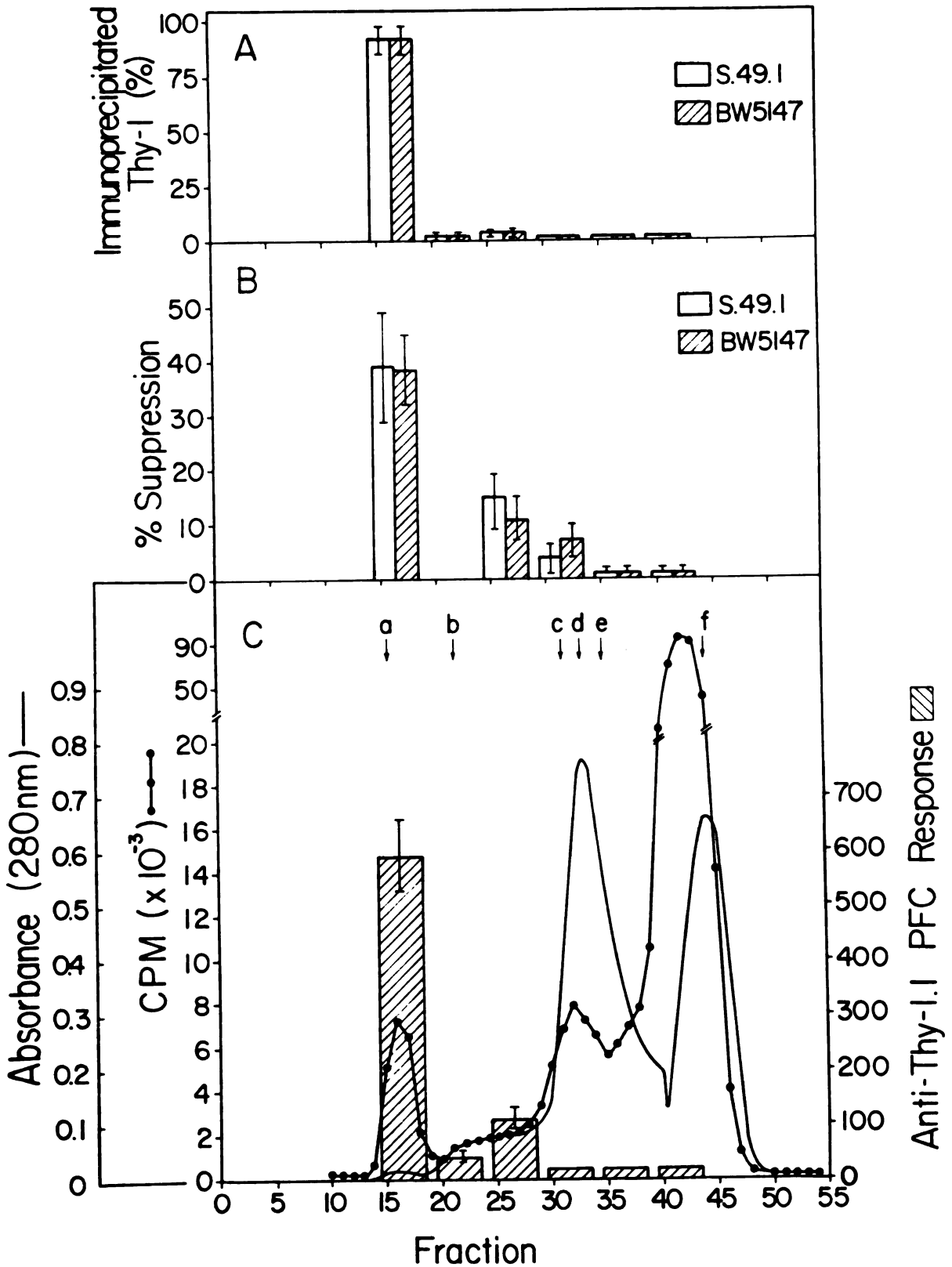
A
S.49.1
BW5147
Immunoprecipitated Thy-1 (%)
100
75
50
25
0
B
S.49.1
BW5147
% Suppression
50
40
30
20
10
0
C
a
b
c
d
e
f
Absorbance (280nm)
CPM (x 10^-3)
Anti-Thy-1.1 PFC Response
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
90
50
20
18
16
14
12
10
8
6
4
2
0
700
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40 45 50 55
Fraction

Figure 1

molecular weight fractions of S.49.1 and BW 5147 culture medium represented 59.5% and 42.1% of the control counts found in unfractionated medium, respectively.

These same pooled fractions were examined for capacity to suppress *in vitro* anti-SRBC responses (Figure 1-B). Significant suppression of the anti-SRBC PFC response was only elicited by fractions 15-18 from both S.49.1 and BW 5147 culture medium. The normal response derived from cultures containing equal quantities of fractionated unconditioned medium was reduced by an average of 39.2% and 38.7% ($p<0.001$ using the Student's t-test), respectively. Although some detectable suppressive activity was demonstrated in fractions 25-28 and 30-33 the degree of suppression was not statistically ($p<0.05$) significant.

Column fractions from supernatant of ^{3}H-glucosamine labeled BW 5147 cells were tested for radioactivity, absorbance (280 nm) and ability to induce anti-Thy-1.1 PFC responses (Figure 1-C). Major peaks of absorbance were detected in fractions 30-38 and 42-48, which co-chromatographed with bovine serum albumin and free amino acids, respectively. Three major peaks of radioactivity were found at fractions 15-18 (I), 30-35 (II), and 39-45 (III). The first peak of radioactivity occurred at the void volume, indicating material of molecular weight greater than 2×10^{6} daltons was released. Peak II contained radiolabeled molecules between 67,000 and 160,000 daltons. Peak III consisted of metabolic products of ^{3}H-glucosamine (33) and some protein or small peptides which were of low molecular weight, approximately $0.5\text{-}5 \times 10^{3}$ daltons. Only two groups of the fractions examined could induce a significant anti-Thy-1.1 PFC response. Pooled fractions 15-18 from peak I were capable of inducing a total of 584

anti-Thy-1.1 plaques while fractions 25-28 induced 108 plaques. Shed Thy-1.1 complexes were therefore primarily of high molecular weight >2 x 10^6 daltons with a small quantity of material estimated to be 3 x 10^5 daltons. S.49.1 (Thy-1.2) culture medium has been examined in an identical manner (33) and most of the antigenic Thy-1.2 material was of high molecular weight greater than 2 x 10^6 daltons. These data indicate that the suppressive activity and Thy-1 antigenic complex released from both lymphoblastoid cells were retained in molecular mixtures greater than two million daltons.

Further verification of the association of Thy-1 antigenicity with modulatory activity results from adsorption of peak I (15-18) fractions with anti-Thy-1 sera (Table III). The mean control anti-SRBC PFC response (3154 PFC/culture) was reduced by the high molecular weight fractions of BW 5147 and S.49.1 culture medium, to 2186 and 1870 PFC/culture (difference between groups 1 vs 2 or 3 $p<0.01$), respectively. Anti-SRBC responses from spleen cell cultures incubated with peak I fraction of normal medium were practically identical to those incubated with only spleen cell culture medium (not presented). Pretreatment of lymphoblastoid peak I fractions (groups 4 and 5) with AKR/J or C3H normal mouse sera did not affect the suppressive activity. Adsorption of peak I material from both S.49.1 or BW 5147 culture with either anti-Thy-1.1 or anti-Thy-1.2 antibodies (group 6-9) resulted in the neutralization of modulatory effects on anti-SRBC PFC responses. BW 5147 peak I material induced a mean 336 PFC/10^7 cells anti-Thy-1.1 response compared to 40 PFC from S.49.1 peak I fractions. C3H normal mouse serum as expected did not influence the anti-Thy-1.1 PFC response. Anti-Thy-1.1 pretreatment of BW 5147 peak I substances reduced the anti-Thy-1.1 PFC response to background levels (45 PFC/10^7

TABLE III

SUPPRESSION OF ANTIBODY RESPONSE AND INDUCTION OF ANTI-THY-1.1 RESPONSES INDUCED BY PEAK I OF CULTURE MEDIUM

	CULTURE[a] FRACTION PEAK I	TREATED WITH[c]	ANTI-SRBC RESPONSE PFC/CULTURE	ANTI-THY-1.1 RESPONSE PFC/10^7 CELLS (AKR TARGET CELLS)
1.	NORMAL MEDIUM	---[d]	3154 ± 162[e]	---[f]
2.	S.49.1 (1.2)[b]	---	2186 ± 96	40 ± 10.4[e]
3.	BW 5147 (1.1)	---	1870 ± 148	336 ± 48.0
4.	S.49.1 (1.2)	AKR/J NMS	2288 ± 139	---
5.	BW 5147 (1.1)	C3H NMS	1978 ± 147	293 ± 36.5
6.	S.49.1 (1.2)	Anti-Thy-1.2	3078 ± 218	---
7.	BW 5147 (1.1)	Anti-Thy-1.2	3352 ± 299	303 ± 52.1
8.	S.49.1 (1.2)	Anti-Thy-1.1	3178 ± 241	---
9.	BW 5147 (1.1)	Anti-Thy-1.1	3030 ± 284	45 ± 13.3

[a]Peak I from Sepharose-6B columns from the indicated normal or lymphoblastoid culture medium was added to BALB/c spleen cell cultures with 2 x 10^7 cells (see Methods). Each culture was tested for both anti-SRBC and anti-Thy-1.1 responses.

[b]Thy-1 allotype in parentheses.

[c]Antisera and NMS were added in same manner as Table II.

[d]No addition.

[e]Means ± standard errors of 5-6 cultures per group.

[f]Not done.

cells). However, BW 5147 peak I induction of PFC response remained unaffected following adsorption with anti-Thy-1.2 antibodies. These data support the previous results suggesting again a close but distinct association between the moieties of a high molecular weight complex which contain Thy-1 antigenicity and suppressor activity.

DISCUSSION

Although depressed antibody responses and other aberrations of the immune response have been repeatedly reported in tumor-bearing animals (3,4,17,18), the mechanisms of these immune dysfunctions are still not understood. Immunosuppressive soluble factors have been characterized in the serum and ascites fluid in a variety of tumor-bearing animals (3,4,17-22). Alexander has proposed that shedding of soluble tumor specific antigens from proliferating neoplastic cells interferes with humoral and cellular immune responses, thus providing an escape mechanism from immune surveillance of the host (3). Several researchers have correlated a high level of soluble tumor associated antigens in the tissue surrounding the tumor and in their serum with increased metastasis and a concomitant reduction in immunological response directed against the growing tumor (3,4,17,41,42).

In this report, significant depression of *in vitro* anti-SRBC plaque forming response was demonstrated by incubation of culture medium from two different T-lymphoblastoid cells with murine spleen cell cultures. The degree of suppression of primary antibody responses was nearly equal for both S.49.1 (Thy-1.2, H-2^d) and BW 5147 (Thy-1.1, H-2^k) conditioned medium whether added to Balb/c (H-2^d) or AKR (H-2^k) spleen cell cultures (Table I). This result suggested that compatibility between the released suppressor factor and spleen cells was not

required for modulation of PFC responses to occur. Similar observations of suppression of *in vitro* antibody responses by tumor ascites fluid or culture medium from neoplastic cells have been observed. Kamo et al. (19) demonstrated that a marked suppression of *in vitro* anti-SRBC PFC responses was induced by incubation of syngeneic spleen cells with ascites fluid or solubilized cell-free homogenates from murine mastocytoma cells. Recently, Huget et al. (21) and Fridman et al. (22) demonstrated that *in vitro* primary antibody responses to SRBC were suppressed by more than 75% following addition of culture supernatants from L1210 mouse lymphoma cells or L-5178-Y mouse thymoma cells, respectively.

Suppressive activity found in both S.49.1 and BW 5147 culture supernatant was abrogated by anti-Thy-1.1 or anti-Thy-1.2 alloantisera suggesting that the suppressor molecule may be associated with the Thy-1 antigenic molecule. Thy-1 associated complexes have been previously reported to be shed from these same tumor cells (33). Since both anti-Thy-1 alloantisera were effective in neutralizing suppressive activity it was possible that these antisera were reactive with released products other than shed Thy-1 molecules. However, when more dilute anti-Thy-1 sera were used the removal of suppressor activity was not complete, but anti-Thy-1 sera were more reactive in removing suppressor activity from culture medium containing the complementary Thy-1 allotype. To demonstrate the presence of shed Thy-1 in lymphoblastoid culture medium and the specificity of anti-Thy-1 alloantisera the *in vitro* anti-Thy-1 PFC plaque assay was performed, using the same spleen cell cultures tested for anti-SRBC PFC responses. Specificity of these anti-Thy-1 sera was demonstrated when only anti-Thy-1.1 and not anti-Thy-1.2 sera could abrogate the induction of

anti-Thy-1.1 PFC responses by BW 5147 (Thy-1.1) culture medium in Balb/c spleen cell cultures. The mechanism by which anti-Thy-1 sera neutralizes suppressive activity or abrogates induction of Thy-1 responses would likely be caused by direct binding to the functional or antigenic portion of the suppressor factor or Thy-1 molecule, respectively, thus masking this site and preventing its interaction with its complementary lymphocyte receptors.

Fractionation of lymphoblastoid culture medium on a Sepharose-6B column was performed in an attempt to dissociate the suppressive activity from the Thy-1 antigenic activity. Significant suppression of anti-SRBC responses was only observed when pooled column fractions containing shed material greater than 2×10^6 daltons (from either S.49.1 or BW 5147 supernatant) were incubated with spleen cell cultures from the beginning of cultivation to time of assay five days later. Normal unconditioned culture medium was also fractionated over the same column and used in identical quantities as lymphoblastoid supernatant when added to spleen cell cultures. This was done to control for non-specific effects on PFC responses by increased concentrations of normal culture medium components.

Evidence has been presented for the protein (43), glycoprotein (44-46) and glycolipid (30,47-50) nature of the Thy-1 antigen. Although the exact biochemical composition of the Thy-1 antigenic molecule remains unclear, there appears to be agreement that carbohydrate molecules are an integral part of this molecule (44-50). Therefore, lymphoblastoid cells were cultured with ^{3}H-glucosamine to radiolabel the carbohydrate portion of the Thy-1 molecule and other cellular glycoproteins and glycolipids (33,37,47,51). The presence of radiolabeled Thy-1.1 and Thy-1.2 antigenic moieties in BW 5147 and S.49.1

culture medium were determined by specific immunoprecipitation. Radiolabeled Thy-1.1 or Thy-1.2 were only detected in the high molecular weight fractions. Shed material from BW 5147 cells consisting of substances greater than two million molecular weight and to a lesser extent substances of approximately 300,000 daltons had the ability to induce anti-Thy-1.1 PFC responses. This result is similar to that previously observed for S.49.1 cells (33). Recently, Kuchel et al. (53) reported that rat thymus Thy-1.1 glycoprotein formed large homogeneous complexes of 300,000 m.w. when deoxycholate was removed from solubilized Thy-1.1 molecules. However, Zwerner et al. (54) have isolated a 25,000 m.w. glycoprotein from BW 5147 lymphoblastoid cells which is capable of absorbing the cytotoxic activity of congenic and heterologous anti-Thy-1.1 sera. Comparison of properties of the suppressor factor and complexes containing Thy-1 antigen which are both shed from BW 5147 and S.49.1 cells suggests that both cells have similar mechanisms of release for these substances. In addition, the fact that the suppressive activity and Thy-1 antigenicity were found predominantly in high molecular weight substances suggests that these molecules are released as membrane complexes.

Preliminary biochemical characterization of Peak I fractions and anti-Thy-1 immunoprecipitates of culture medium from S.49.1 and BW 5147 cells labeled with radioactive protein, carbohydrate and lipid precursors on SDS-PAGE has demonstrated the presence of at least four proteins or glycoproteins plus phospholipids and glycolipids (52). This observation indicates that the Thy-1 antigen and suppressor factor may be derived from the same macromolecule, which is complex in nature and has all the necessary components representative of a shed membrane fragment. Recent electron microscopic examination of

shed material from murine mammary tumor virus infected tumor cells and ATP depleted human erythrocytes demonstrated that membrane vesicles resembling liposomes were released into the culture medium, containing several cell surface components (28,29).

The presence of Thy-1 associated complex and suppressor factor(s) in culture medium after 40 hours of incubation is primarily a dynamic metabolic process. The lymphoblastoid cells were greater than 95% viable during the entire cultivation period and had doubled in population after 24 hours. Active biosynthesis and release of radiolabeled Thy-1 was demonstrated in a previous report (33). Therefore, the appearance of suppressor factor or Thy-1 complexes was due to an active metabolic process and their presence in culture medium by cell disintegration was minimal in our culture system.

A partially purified preparation containing both modulatory factor and Thy-1 antigenic macromolecules was tested for its ability to simultaneously suppress anti-SRBC PFC responses and induce specific anti-Thy-1.1 PFC responses. In these experiments suppressive activity was abrogated by both anti-Thy-1 alloantisera, while anti-Thy-1.1 PFC responses were selectively neutralized by anti-Thy-1.1 sera pretreatment of BW 5147 culture medium, verifying earlier results using unfractionated culture media. The inability of AKR or C3H normal mouse sera to abrogate suppressor activity in these experiments indicates that anti-Thy-1 sera selectively reacts with the suppressor substance to eliminate its biological function. Furthermore, this observation and the ability of partially purified high molecular weight substances to suppress antibody responses in the presence of fresh culture medium strongly argues against the possibility that exhaustion of essential nutrients caused suppression. Whole culture

medium and high molecular weight fractions did not demonstrate any cytotoxic effects as evidenced by the similar population size and viability of spleen cells after five days of cultivation.

Identification and characterization of factors produced by tumors, which inhibit antibody responses, has received limited investigations. Virus encoded products from murine leukemia virus infected tumor cells have been shown to impair humoral responses (16,55). Kamo et al. (19,56) identified a soluble suppressor factor which was greater than 12,000 m.w. and heat sensitive at 56°C for 30 minutes. Immune responsiveness could be restored to suppressed spleen cell cultures by addition of SRBC stimulated T-cells, suggesting to these investigators that helper T-cells were being affected. Huget et al. (21) determined that the suppression of PFC responses was caused in part by a direct cytotoxic effect on lymphocytes and macrophages from a heat labile non-dialyzable substance(s). Preincubation of spleen cells for one hour with 20% L1210 lymphoma culture medium suppressed greater than 95% of antibody response, which suggested to these authors that non-proliferating T-cells were the target cell (21). The L-5178-Y thymoma-suppressor factor was absorbed by IgG coated sepharose columns and its suppressive activity was found in 300,000 and 140,000 substances (57). A similar suppressor factor termed immunoglobulin binding factor (IBF) was previously reported by Fridman et al. (5) to be released from alloantigen activated T-cells. This factor binds to the Fc portion of IgG and suppresses direct PFC responses to SRBC and T independent antigens. This lymphoma suppressor factor was most inhibitory to production of PFC responses when added late to spleen cultures, suggesting IBF may act on the final differentiation steps of antigen stimulated precursor cells to plasma

cells (22,57). Demonstration that the IBF factor was released by lymphoma cells and antigen stimulated T-cells suggests that some tumor cells have similar functions as normal cells.

The relationship between the suppressive activity and the Thy-1 antigenic moiety has not been resolved. The observation that only anti-Thy-1.1 alloantisera abrogated anti-Thy-1.1 PFC responses while both anti-Thy-1.1 and anti-Thy-1.2 abrogate the modulatory activity in either S.49.1 or BW 5147 culture medium suggests that a molecule other than the Thy-1.2 or Thy-1.1 antigenic moieties on shed substances embodies the suppressor activity. Since both anti-Thy-1 alloantisera equally eliminate suppression by shed material of either cell type, there must be a substance (presumably antibodies) in both antisera that can react with the suppressor factor from either cell. To explain the production of such antibodies in mice injected with thymocytes from a strain of histocompatible mice (AKR and C3H are both $H\text{-}2^k$) would suggest this method produces antibodies that were cross-reactive with the suppressor factor and Thy-1 molecules, which are encoded by genes within the H-2 complex. It is also possible that other antibodies were produced with different specificites directed against molecules encoded by genes outside the H-2 region which may react with the suppressor factor such as the M locus differences between these strains. The Thy-1 antigenic moiety may not represent just a single determinant but a complex molecule having several different structures associated with it. There is strong evidence that Thy-1 antigenicity is retained on both glycoproteins (44-46) and glycolipids (47-50) while the necessity of lipid moieties for antigenic integrity has also been demonstrated (45). Several researchers have observed three different antigenic specificites on the same Thy-1

molecule using xenogeneic, and allogenic and congenic anti-Thy-1 sera (45,46,58). The gene(s) which encodes for the Thy-1 molecule may also determine the modulatory activity or be linked to genes that do. The genes that regulate anti-Thy-1 responses may simultaneously regulate anti-suppressor factor activity, thus inducing anti-Thy-1 and anti-suppressor factor antibodies simultaneously. Zaleski and Klein have demonstrated there are at least two genes that regulate the anti-Thy-1 response one found inside the H-2 region, the other gene is outside the H-2 complex and postulate Thy-1 is recognized in association with H-2 antigens (36). Based on these proposals, a suppressor factor with identical or similar composition could exist on each lymphoblastoid cell and be equally reactive with both anti-Thy-1.2 and anti-Thy-1.1 alloantisera. If the carbohydrate moiety of Thy-1 is the antigenic portion, as several researchers have suggested (44,47-49,59,60), then the possibility of a common backbone for Thy-1.1 and Thy-1.2 antigen or a molecule closely associated with Thy-1 antigenic moiety could elicit antibody responses not only against the Thy-1 antigenic moiety but also against those structures intimately associated with Thy-1 (such as the modulatory factor). In addition, exhaustively absorbed rabbit anti-BA-Thy-1 cannot distinguish between the two Thy-1 allotypes and lyses thymocytes of either Thy-1 allotype in the presence of complement, suggesting that there is a common structure present in both Thy-1.1 and Thy-1.2 antigens or on both these thymocyte cell surfaces (61).

Previous studies in our laboratory demonstrated the intimate association between Thy-1 antigenicity and suppressor activity found in antigen-activated T-cell culture medium and in G_{M1} ganglioside (formulated in cholesterol-lecithin liposomes) extracted from this

medium or from mouse brain (30,31). Although these modulatory factors were reactive with and neutralized by only anti-Thy-1 sera directed against the Thy-1 allotype they carried, there has been cross-reactivity observed in binding assays such as hapten inhibition (30). The mechanism by which lymphoblastoid culture supernatant suppresses anti-SRBC is still being investigated. The apparent close association of the Thy-1 molecule with the suppressor factor suggests that the mechanism of suppression of antibody responses may be similar to that observed with Thy-1 associated modulatory factors (30,31). These factors delay the appearance of the normal anti-SRBC response by temporarily inhibiting the final stages of B-cell differentiation into plasma cells.

Speculation as to the role for Thy-1 released from a growing lymphoma *in vivo* may be the modulation of antibody responses directed against the tumor, similar in manner as observed in *in vitro* modulation of anti-SRBC responses (30,31). Gordon et al. (62) found a tumor specific transplantation antigen on the surface of murine lymphoma cells and in ascites fluid capable of absorbing anti-Thy-1.2 cytotoxic capacity against Thy-1.2 bearing thymocytes. This isolated antigen was greater than 2×10^5 daltons and probably represented an altered form of Thy-1. When injected *in vivo*, this substance could prolong survival of mice, which later received a tumor challenge, presumably by immunizing mice against this tumor associated antigen (62). We have also found that coating S49A tumor cells (*in vivo* tumor from which S.49.1 was derived) with rabbit anti-BA-Thy-1 prior to challenge, or injecting this antiserum into the vicinity of tumor growth within two days after tumor challenge, would prevent tumor

growth in the great majority of challenged mice (manuscript in preparation).

In summary, these studies on the modulation of antibody responses by conditioned media from T-lymphoblastoid cells demonstrated that these cells shed high molecular weight complexes which contain suppressor activity and the Thy-1 antigen. The association between modulatory activity and the Thy-1 antigenicity was investigated by pretreatment of lymphoblastoid culture supernatant with anti-Thy-1.1 and anti-Thy-1.2 sera, resulting in the abrogation of suppressor activity (irrespective of Thy-1 allotype), but specific neutralization of anti-Thy-1 PFC responses. The evidence presented in this report suggests that 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 closely associated with it. Biological significance of these events is presently being investigated to determine the role of these shed Thy-1 associated complexes in immune surveillance of tumor growth.

REFERENCES

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

2. Katz, B. H. and D. Armerding. 1976. The role of histocompatibility gene products in lymphocyte triggering and differentiation. Fed. Proc. 35:2053.

3. 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.

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

5. Gisler, R. H. and W. H. Fridman. 1975. Suppression of *in vitro* antibody synthesis by immunoglobulin-binding factor. J. Exp. Med. 142:507.

6. Taniguchi, M., K. Hayakawa and T. Tada. 1976. Properties of antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. II. *In vitro* activity and evidence for the I region gene product. J. Immunol. 116:542.

7. Tadakuma, T., A. L. Kuhner, R. R. Rich, J. R. David and C. W. Pierce. 1976. Biological expressions of lymphocyte activation. V. Characterization of a soluble immune response suppressor (SIRS) produced by concanavalin A-activated spleen cells. J. Immunol. 117:323.

8. Kapp, J.A., C. W. Pierce, F. DeLaCroix and B. Benacerraf. 1976. Immunosuppressive factor(s) extracted from lymphoid cells of non-responder mice primed with L-glutamic acid ^{60}-L-alanine^{3-}-L-

tyrosine^{10} (GAT). I. Activity and antigenic specificity. J. Immunol. 116:305.

9. Douglas, G. N. and A. S. Rubin. 1977. Non-specific suppression of the initiation of the immune response to a heterologous immunogen by supernatants from specifically stimulated, primed lymphoid cells. Immunology 32:669.

10. Gorczynski, R. M., R. G. Miller, and R. A. Phillips. 1972. Initiation of antibody production to sheep erythrocytes *in vitro*: replacement of the requirement for T-cells with a cell-free factor isolated from cultures of lymphoid cells. J. Immunol. 108:547.

11. Watson, J. 1973. The role of humoral factors in the initiation of *in vitro* primary immune responses. III. Characterization of factors that replace thymus-derived cells. J. Immunol. 111:1301.

12. Thomas, D. W., W. K. Roberts and D. W. Talmage. 1975. Regulation of the immune response: Production of a soluble suppressor by immune spleen cells *in vitro*. J. Immunol. 114:1616.

13. Eshhar, Z., D. Armerding, T. Waks, and D. H. Katz. 1976. Activation of T and B lymphocytes *in vitro*. V. Cellular locus, metabolism and genetics of induction and production of the allogeneic effect factor. J. Immunol. 119:1457.

14. Taussig, M. J. 1974. T-cell factor which can replace T-cells *in vivo*. Nature 248:234.

15. Tada, T., M. Taniguchi, and T. Takemori. 1975. Properties of primed suppressor T-cells and their products. Transplant. Rev. 26:106.

16. Specter, S., C. Patel, and H. Friedman. 1976. Immunosuppression induced *in vitro* by cell-free extracts of Friend leukemia virus-infected splenocytes. J. Natl. Can. Inst. 56:143.

17. Gorczynski, R. M., D. G. Kilburn, 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.

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

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

20. 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.

21. Huget, R. P., H.-D. Flad, and H. G. Opitz. 1977. Suppression of *in vitro* primary immune response by L1210 cells and their culture supernatant: evidence for cytotoxic effects. Cell Immunol. 29:210.

22. Fridman, 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.

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

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

25. Ramseier, H. 1975. Spontaneous release of T-cell receptors for alloantigens. II. Induction of antibodies to T-cell receptors. Eur. J. Immunol. 5:23.

26. Kapeller, M., U. M. Plesser, N. Kapeller, 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.

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

28. Calafat, J., J. Hilgers, W. J. Van Blitterswijk, 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.

29. Lutz, H. V., S.-C. Liv, and J. Palek. 1977. Release of Spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of Spectrin-free vesicles. J. Cell Biol. 73:548.

30. 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.

31. 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.

32. 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.

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

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

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

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

37. Reif, A. E. and J. M. Allen. 1966. Mouse thymic isoantigen. Nature (Lond.) 209:521.

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

39. Taylor, G. M. and J. Bennett. 1973. A modified plaque test for the detection of cells forming antibody to alloantigens. J. Immunol. Meth. 2:213.

40. Reiland, J. 1971. Gel filtration. In *Methods in Enzymology,* Vol. 22. Edited by J. J. Jakoby, Academic Press, New York, p. 287.

41. Nowotony, A., J. Groshman, A. Abdelnoor, N. Rote, C. Yang and R. Waltersdoff. 1974. Escape of TA3 turnover from allogeneic immune rejection: theory and experiments. Eur. J. Immunol. 4:73.

42. Thomson, D. M. 1975. Soluble tumor-specific antigen and its relationship to tumor growth. Int. J. Can. 15:1016.

43. Kucich, U. N., J. C. Bennett, and B. J. Johnson. 1975. The protein nature of Thy-1.2 alloantigen as expressed by the murine lymphoblastoid line S.49.1 TB.2.3. J. Immunol. 115:626.

44. 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 (Lond.) 256:652.

45. 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.

46. Williams, A. F., A. N. Barclay, M. Letarte-Muirhead, and A. J. Morris. 1977. Rat Thy-1 antigens from thymus and brain: their tissue distribution, purification, and chemical composition. Cold Spring Harbor Symp. Quant. Biol. 41:51.

47. 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.

48. Esselman, W. J. and H. C. Miller. 1974. Brain and thymus lipid inhibition of anti-brain associated θ cytotoxicity. J. Exp. Med. 139:445.

49. 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.

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

51. Van Eijk, R. V. W. and P. F. Muhlradt. 1977. Carbohydrate incorporation in plasma membranes of mouse thymocytes stimulated by concanavalin A. Eur. J. Biochem. 78:41.

52. Freimuth, W. W. 1978. Ph.D. Dissertation, Michigan State University.

53. Kuchel, P. W., D. G. Campbell, A. N. Barclay and A. F. Williams. 1978. Molecular weights of the Thy-1 glycoproteins from rat thymus and brain in the presence and absence of deoxycholate. Biochem. J. 169:411.

54. Zwerner, R. K., P. A. Barstad and R. T. Acton. 1977. Isolation and characterization of murine cell surface components. I. Purification of milligram quantities of Thy-1.1. J. Exp. Med. 146:986.

55. Dent, P. B. 1972. Immunodepression by oncosenic viruses. Progr. Med. Virol. 14:1-35.

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

57. Neauport-Sautes, C., and W. H. Fridman. 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.

58. 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.

59. Trowbridge, I. S. and R. Hyman. 1975. Thy-1 variants of mouse lymphomas: biochemical characterization of the genetic defect. Cell 6:279.

60. 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 S.49.1 TB.2.3. J. Immunol. 116:1669.

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

62. Gardon, W. C., F. S. Baechtel, G. Goetz, and M. D. Prager. 1977. Immunogenicity of solubilized tumor antigen extracted from P1798 murine lymphoma cells or isolated from tumor-bearer ascites fluid and reactivity with anti-Thy-1.2 antiserum. Int. J. Can. 19:692.

APPENDIX

APPENDIX

FURTHER BIOCHEMICAL CHARACTERIZATION OF THY-1 ASSOCIATED COMPLEXES SHED FROM S.49.1 AND BW 5147 LYMPHOBLASTOID CELLS

Introduction

The composition of macromolecules and membrane complexes which are released from normal and neoplastic cells has only been partially characterized. Previous research has focused on the isolation of membrane-associated constituents, like glycoprotein, glycolipids, protein, or lipids, from culture medium or serum of tumor bearing mice (1). The exact nature of material which is shed from the membrane is still open to investigation. Recent models of shedding suggest that a pinching off of microvilli precedes the release of this membrane material into the surrounding medium (1-3). Shed substances are believed to contain lipid bilayer complexes and select membrane constituents together in the form of liposomes (1-3). This proposal has been supported by recent electron microscopic studies of shed material from tumor cells (4) and human erythrocytes (5).

Studies on metabolism of the cell surface component Thy-1 antigen have demonstrated that this molecule is rapidly released from unstimulated thymocytes (3). Vitetta et al. (3,6) reported that the nature of the Thy-1 antigenic molecule was a ^{3}H-galactose-labeled substance of 35,000 m.w. However, the exact nature of the Thy-1 antigenic molecule remains unresolved; as evidence for its protein (7), glycoprotein (8-10), glycolipid (3,6,11-14) properties have been reported. In this communication a further biochemical characterization of the soluble complexes shed from S.49.1 and BW 5147 lymphoblastoid cells is reported. Incorporation of radiolabeled precursors of protein, glycoprotein, glycolipid and lipid into these shed complexes was used to determine their composition. The individual components were analyzed by sodium dodecyl sulfate

polyacrylamide gel electrophoresis. Results from these studies suggested that high molecular weight shed substances were heterogeneous in composition and a glycolipid and/or glycoprotein (∿53,000 daltons) may contain the Thy-1 antigenic moiety. Further verification of the glycoprotein and/or glycolipid nature of shed Thy-1 were found in deoxycholate column chromatography and anti-G_{M1} ganglioside adsorption of lymphoblastoid culture medium.

Materials and Methods

Cells and Media. AKR (Thy-1.1, H-2^k) and CBA (Thy-1.2, H-2^d) mice 8-16 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME). Spleens and thymuses were obtained aseptically from mice for culture in Marbrook culture vessels. Preparation of cell suspensions and culture medium was described in detail in a previous article (15).

Lymphoblastoid Cells and Supernatants. Murine lymphoblastoid cell lines S.49.1 (Thy-1.2, H-2^d) and BW 5147 (Thy-1.1, H-2^k) were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat inactivated fetal calf serum (FCS) and antibiotics (D-MEM/FCS). A complete description of the culture medium and manner of collection or concentration of culture medium is explained fully in previous articles (15,16).

Antisera. The preparation and titers of anti-Thy-1.2 and anti-Thy-1.1 alloantisera and goat anti-rabbit Ig sera have been described in previous articles (15,16). Anti-G_{M1} ganglioside sera were prepared as previously described (12). Dutch Belt rabbits were injected with purified C3H mouse brain ganglioside containing

100-500 µg of ganglioside dissolved in 1 ml of 0.1M NaCl containing 1 mg of bovine serum albumin. This solution was emulsified in Freund's complete adjuvant and injected in six subcutaneous sites. Two to three injections 1 month apart usually resulted in antisera reactive with primarily G_{M1} ganglioside and to a lesser extent G_{D1B} ganglioside (12).

Radiolabeling of Lymphoblastoid Cells. Membrane and cell associated components of S.49.1 and BW 5147 cells were labeled during incubation in D-MEM/FCS with 1000 mg/l of glucose instead of 4500 mg/l plus [6-^{3}H(N)]-D-glucosamine-HCl (29.0 Ci/mmole) or (1-^{14}C)-D-glucosamine-HCl (51.8 mCi/mmole) for 24 hours (New England Nuclear, Boston, MA). S.49.1 cells were labeled with [4,5-^{3}H]-L-leucine (15 Ci/mmole) (NEN) in leucine-free D-MEM/FCS for 10 hours. S.49.1 cells were labeled with [methyl-^{3}H]-choline chloride (13 Ci/mmole) (Amersham, Inc., England) in choline chloride-free D-MEM/FCS during a 10 hour incubation period. All the radioactive precursors were added to culture medium at the concentration of 2 µCi/ml of medium. Cells for culture were washed once, then incubated in fresh D-MEM/FCS plus radiolabeled precursor at a concentration of 10^{6} cells/ml in a humid 8% CO_2 atmosphere at 37°C. After 10 or 24 hours the cells were washed three times in D-MEM plus 5% FCS and resuspended in complete culture medium at a concentration of 8 x 10^{5} cells/ml in roller bottles containing 250 ml of the radiolabeled cell suspension. At the end of the 40 hr incubation period, cultures were removed from the incubator and viable cell counts were determined. Viabilities were greater than 95% and the concentration of cells had

doubled during 24.5 hours of incubation. Culture medium was separated from cells by centrifugation (1600 x g) for 10 min.

Immunoprecipitation of Cell-Free Culture Medium and Column Fractions. Radiolabeled lymphoblastoid culture medium (5.0 ml) (or pooled column fractions, 4.0 ml) were processed by a double antibody immunoprecipitation technique previously described (15,16).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was used to analyze the composition and molecular weight of components found in Thy-1 associated complexes. Peak I material (>2 x 10^6 daltons) from culture medium of ^{3}H-glucosamine, ^{3}H-leucine, and ^{3}H-choline labeled S.49.1 cells and ^{3}H-glucosamine labeled BW 5147 cells was concentrated 10-fold on CF-50A centriflo membrane cones (Amicon Corp., Lexington, MA). Anti-Thy-1 immunoprecipitates were washed three times in phosphate buffered saline (PBS) and excess PBS was removed so that approximately 100 µl remained with the pellet. Before addition of sample buffer to the Peak I material, the precipitates were sonicated vigorously for 1 minute or until precipitates were mostly dissolved on a bath sonicator. Precipitates were further sonicated for 2-5 seconds by a probe sonicator to increase solubilization of precipitates. The sonicated samples were added in equal quantity to 2X sample buffer containing 2-mercaptoethanol and boiled for 5 min. The reagents, preparation of 10% polyacrylamide gels, apparatus, and procedures for electrophoresis and staining are identical to those described in detail by Porzio and Pearson (17). Some of the molecular weight standards used in these studies were myofibril proteins, primarily myosin (200,000 daltons). The other standards were bovine serum albumin (67,000

daltons), soybean trypsin inhibitor (23,000 daltons) and ^{3}H-G_{M1}-ganglioside (1640 daltons). The gels were cut into 1 mm slices and 2 slices were placed into 6 ml of a toluene based scintillation fluid containing 5% NCS tissue solubilizer and 1% of a 4N NH_4OH solution. After overnight incubation at 37°C the radioactivity was counted directly.

Gel Filtration of Radiolabeled Lymphoblastoid Cell Culture Supernatant. High molecular weight material >2 x 10^6 daltons (Peak I) from radiolabeled S.49.1 and BW 5147 cell-free culture supernatants was obtained by gel filtration over a Sepharose-6B column as previously described in the first two articles. Peak I fractions from ^{3}H-leucine and ^{3}H-glucosamine labeled S.49.1 cells were placed on a Sephacryl-S-200 column equilibrated and run with 1% deoxycholate (DOC) in PBS (pH 7.2). The column was calibrated by the following five molecular weight standards: 1) blue dextran (>2 x 10^6 daltons), 2) bovine serum albumin (67,000 daltons), 3) soybean trypsin inhibitor (23,000 daltons), 4) lysozyme (14,000 daltons), and 5) phenol red (376 daltons). Six milliliter samples of radiolabeled Peak I were applied and 2.1 ml fractions were collected. Pooled fractions were precipitated with 95% ethanol (16 hours at -10°C) twice and washed twice with cold ethanol. The precipitates were solubilized in 2 ml and dialyzed in a large volume of PBS overnight and stored in antibiotics. This procedure of ethanol precipitation removes DOC and extracts primarily glycoprotein and glycolipids (10). These samples were then tested for their ability to induce secondary anti-Thy-1.2 PFC responses.

Anti-Thy-1 Plaque Assay. The *in vitro* induction of primary and secondary responses to Thy-1 were measured by a plaque forming cell assay which has previously been described in detail (15,18-20). For secondary anti-Thy-1.2 responses, AKR mice were primed by immunization with 4 x 10^7 CBA thymus cells in Eagle's MEM-Hanks Salts (Grand Island Biological Co.) intravenously two to four weeks before use. Spleen cells from primed mice were processed in a similar manner as untreated spleen cells. Secondary anti-Thy-1.2 PFC responses were used in measuring the ability of DOC column fractions to produce anti-Thy-1.2 PFC responses, because it was more sensitive than primary responses (19,20).

Results

Culture medium from 40 hour cultures of ^{3}H- or ^{14}C-glucosamine, ^{3}H-leucine or ^{3}H-choline labeled S.49.1 or BW 5147 lymphoblastoid cells was placed on a Sepharose-6B column to separate Thy-1 containing substances. These radioactive compounds are precursors for glycolipids, glycoproteins, proteins and lipids, respectively (21), and were utilized to detect the association of any of these compounds with Thy-1 antigenic complexes. ^{3}H- or ^{14}C-glucosamine labeled products (groups 1, 2 and 5) were incorporated in greater quantities into the high molecular weight fractions (>2 x 10^6 daltons) termed peak I (15), than ^{3}H-leucine or ^{3}H-choline labeled products (Table I).

The quantity of radiolabeled Thy-1 associated complexes immunoprecipitation in peak I samples and unfractionated culture medium were measured. Peak I substances contained a much higher percentage of Thy-1 associated precipitable counts (10-60 fold increase in

Table I

Immunoprecipitation of the Thy-1 Antigen in the Peak I Fraction of Culture Media from Lymphoblastoid Cells Radiolabeled with Protein, Carbohydrate and Lipid Precursors

	Radiolabeled Culture Medium[a]	% Peak I cpm / Total cpm	% Thy-1 assoc. cpm in: Total cpm	% Thy-1 assoc. cpm in: Peak I cpm	Thy-1 assoc. cpm % Peak I / Total	% Thy-1 in Peak I / Thy-1 in all fractions
1.	S.49.1[b] (^{3}H-glucosamine)	8.68	6.2	34.0	41.1	97.0
2.	S.49.1 (^{14}C-glucosamine)	4.70	1.2	15.3	59.5	92.0
3.	S.49.1 (^{3}H-leucine)	1.49	0.37	9.9	39.4	94.4
4.	S.49.1 (^{3}H-choline)	0.45	0.20	12.1	27.6	93.4
5.	BW 5147 (^{3}H-glucosamine)	4.13	0.80	8.2	42.1	94.0

[a]Culture medium obtained after 31-40 hours of incubation from lymphoblastoid cells labeled with the designated radioactive precursor (see Methods).

[b]Group I was a separate experiment from groups 2-5 whose culture medium was centrifuged at 10,000 x g before immunoprecipitation.

groups 2-5) in comparison to that found in whole medium. This result suggests a significant enrichment of Thy-1 macromolecules in Peak I substances isolated from culture medium of lymphoblastoid cells labeled with any of the radioactive precursors tested. There was not any major difference in the percentage of Thy-1-associated radioactivity detected in Peak I material from S.49.1 cells labeled with ^{14}C-glucosamine (15.3%), ^{3}H-leucine (9.9%) or ^{3}H-choline (12.1%) and BW 5147 cells labeled with ^{3}H-glucosamine (8.2%). The amount of Thy-1 associated radioactivity recovered in peak I column fractions from unfractionated culture medium was similar for S.49.1 and BW 5147 cells. In previous articles (15,16), data were presented that showed only high molecular weight fractions of culture medium from ^{14}C- or ^{3}H-glucosamine labeled S.49.1 or BW 5147 cells contained the only detectable radioactivity following anti-Thy-1 immunoprecipitation of these fractions. Pooled fractions containing material of different molecular weights (see Figure 1-C, ref. 16) of culture medium from ^{3}H-leucine and ^{3}H-choline labeled S.49.1 cells were tested for Thy-1 precipitable radioactivity. Thy-1 associated complexes in Peak I contained 94.4% and 93.4% of the total Thy-1 associated ^{3}H-leucine and ^{3}H-choline radioactivity, respectively, which was detected in all the fractions tested. These data suggest that the high molecular weight macromolecules (>2 x 10^{6} daltons) found in Peak I fractions contain practically all of the Thy-1 immunoprecipitable radioactivity in BW 5147 or S.49.1 culture medium, irrespective of the radiolabeled precursor tested. Furthermore, these studies indicate that Thy-1 associated complexes contain significant quantities of glycoprotein and/or glycolipid, protein and lipids.

The composition of Peak I material from culture medium of radiolabeled S.49.1 and BW 5147 cells was examined by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) of these substances (Figure 1). ^{3}H-leucine labeled Peak I (S.49.1) material had a broad spectrum of radioactivity throughout the gel with major radioactive peaks occurring in substances of approximately 250,000, 75,000, 39,000 and 17,000 daltons (Figure 1A). Polyacrylamide gels of ^{3}H-glucosamine Peak I (S.49.1) macromolecules showed four major peaks of radioactivity in molecules estimated to be 130,000, 75,000 and 55,000 daltons and a low molecular weight band co-migrating with ^{3}H-G_{M1} ganglioside (Figure 1B). The presence of radioactivity at the top of the gel was variable. High molecular weight material of Peak I labeled with ^{3}H-choline was found in only one major peak of low molecular weight co-migrating with the radioactive glycolipid standard (Figure 1C). ^{3}H-glucosamine labeled Peak I material from BW 5147 cells had some radioactive high molecular weight substances >180,000 and major peaks of radioactivity associated with molecules of approximately 95,000, 75,000, and 53,000 daltons and a low molecular weight compound migrating with radioactive G_{M1} ganglioside (Figure 1D). These results demonstrate that Peak I material shed from S.49.1 and BW 5147 cells is a heterogeneous complex containing protein, glycoprotein, glycolipid and lipid components indicative of a membrane fragment.

Culture medium from ^{3}H-leucine, ^{3}H-choline, and ^{3}H-glucosamine labeled S.49.1 (Thy-1.2) cells was immunoprecipitated with anti-Thy-1.2 and anti-Thy-1.1 alloantisera and excess goat anti-mouse IgG. The composition of the radiolabeled molecules in these precipitates was analyzed by SDS-PAGE (Figure 2). Anti-Thy-1.2

Figure 1. Analysis of high molecular weight (>2 x 10^6 daltons) material (Peak I), shed from radiolabeled S.49.1 and BW 5147 lymphoblastoid cells, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Peak I material from S.49.1 cells labeled with: A, ^{3}H-leucine; B, ^{3}H-glucosamine; and C, ^{3}H-choline were examined. In addition, D, Peak I material from BW 5147 cells labeled with ^{3}H-glucosamine was studied. The molecular weight markers represented are the following: a, myosin (200,000 daltons); b, bovine serum albumin (67,000 daltons); c, soybean trypsin inhibitor (23,000 daltons); and d, ^{3}H-G_{M1} ganglioside (1640 daltons); TD represents the position of the Pyronin Y tracking dye.

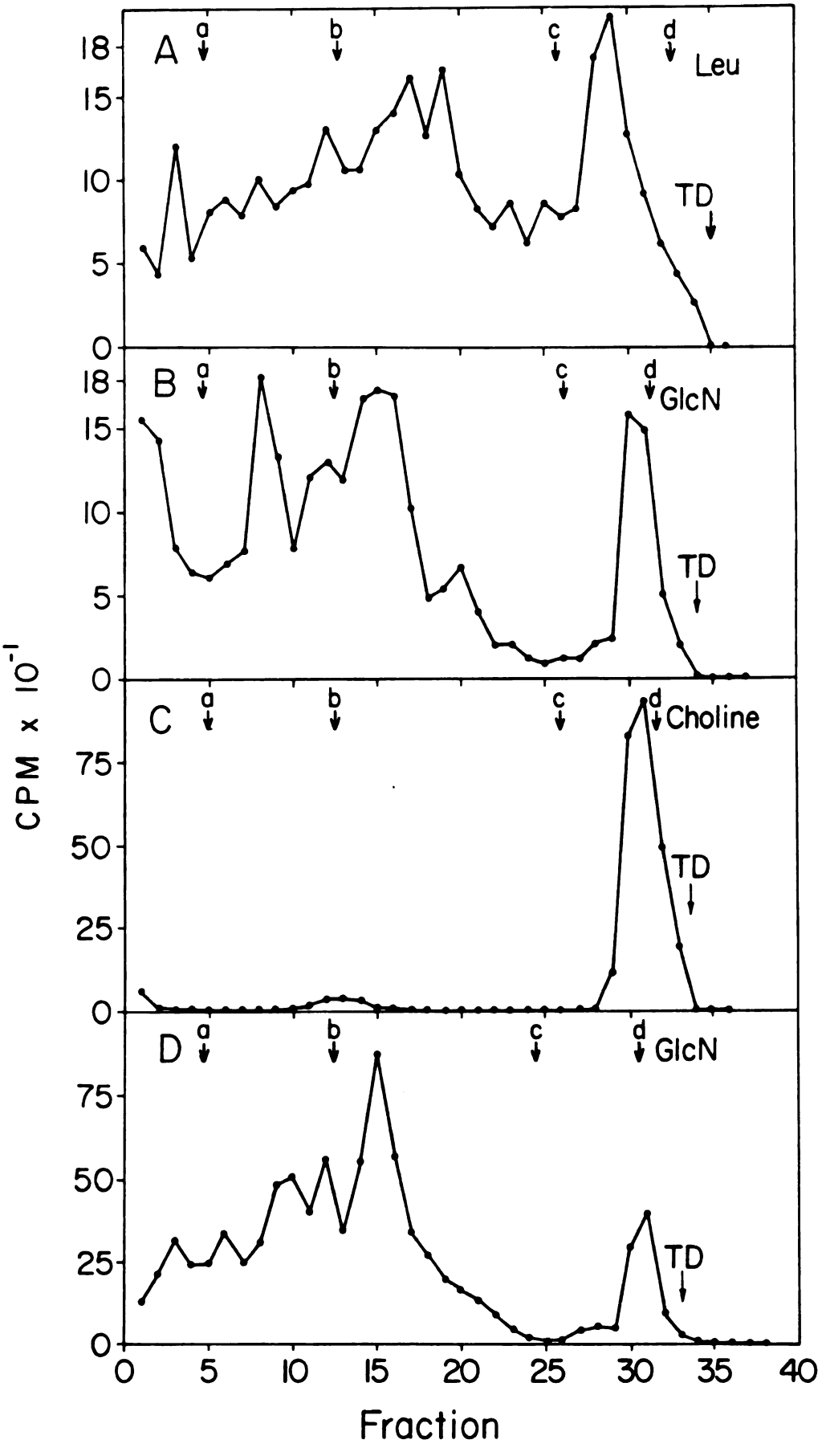
A
B
C
D
a
b
c
d
Leu
GlcN
Choline
TD
CPM x 10⁻¹
Fraction
0
5
10
15
18
25
50
75
20
30
35
40

Figure 1

precipitates of ^{3}H-leucine labeled shed Thy-1.2 complexes contained three peaks of radioactivity, one at the very top of the gel (which was variable in appearance) and molecules of approximately 51,000 and 19,000 daltons (Figure 2A). Shed material precipitated by anti-Thy-1.1 serum contained only one major peak of activity at 19,000 daltons, suggesting that the high molecular weight substance and the 50,000 daltons substance were specifically associated with Thy-1.2 complexes. ^{3}H-choline labeled shed macromolecules immunoprecipitated with anti-Thy-1.2 contained only one broad peak of radioactivity of low molecular weight migrating with radiolabeled glycolipid (Figure 2B). Only a small amount of radioactivity was found in low molecular weight substances in anti-Thy-1.1 precipitates suggesting that a significant quantity of lipids were contained in shed Thy-1.2 associated substances. Anti-Thy-1.2 precipitates of culture medium from ^{3}H-glucosamine labeled S.49.1 cells was composed of three peaks of radioactivity associated with molecules of about 105,000 and 34,000 daltons and low molecular weight molecules migrating with the glycolipid standard (Figure 2C). Radioactivity observed in the top of the gel was variable and probably due to large insolubilized complexes which usually penetrate the gel but may also be washed away. A major band of radioactivity was found in a fraction with molecules estimated to be 54,000 daltons. There were no significant radioactive peaks associated with anti-Thy-1.1 precipitates suggesting that the above-mentioned substances were specifically associated with shed Thy-1.2 complexes. Similar results were obtained when ^{3}H-glucosamine labeled Peak I material of S.49.1 cells was immunoprecipitated (Figure 2D). Radioactivity was found in molecules of approximately 115,000 and 56,000 daltons and

Figure 2. SDS-PAGE analysis of anti-Thy-1.2 (●——●) and anti-Thy-1.1 (O——O) immunoprecipitates of culture medium from S.49.1 cells radiolabeled with A, ^{3}H-leucine; B, ^{3}H-choline; and C, ^{3}H-glucosamine; D, Peak I material from ^{3}H-glucosamine labeled S.49.1 cells. The molecular standards are identical to those in Figure 1.

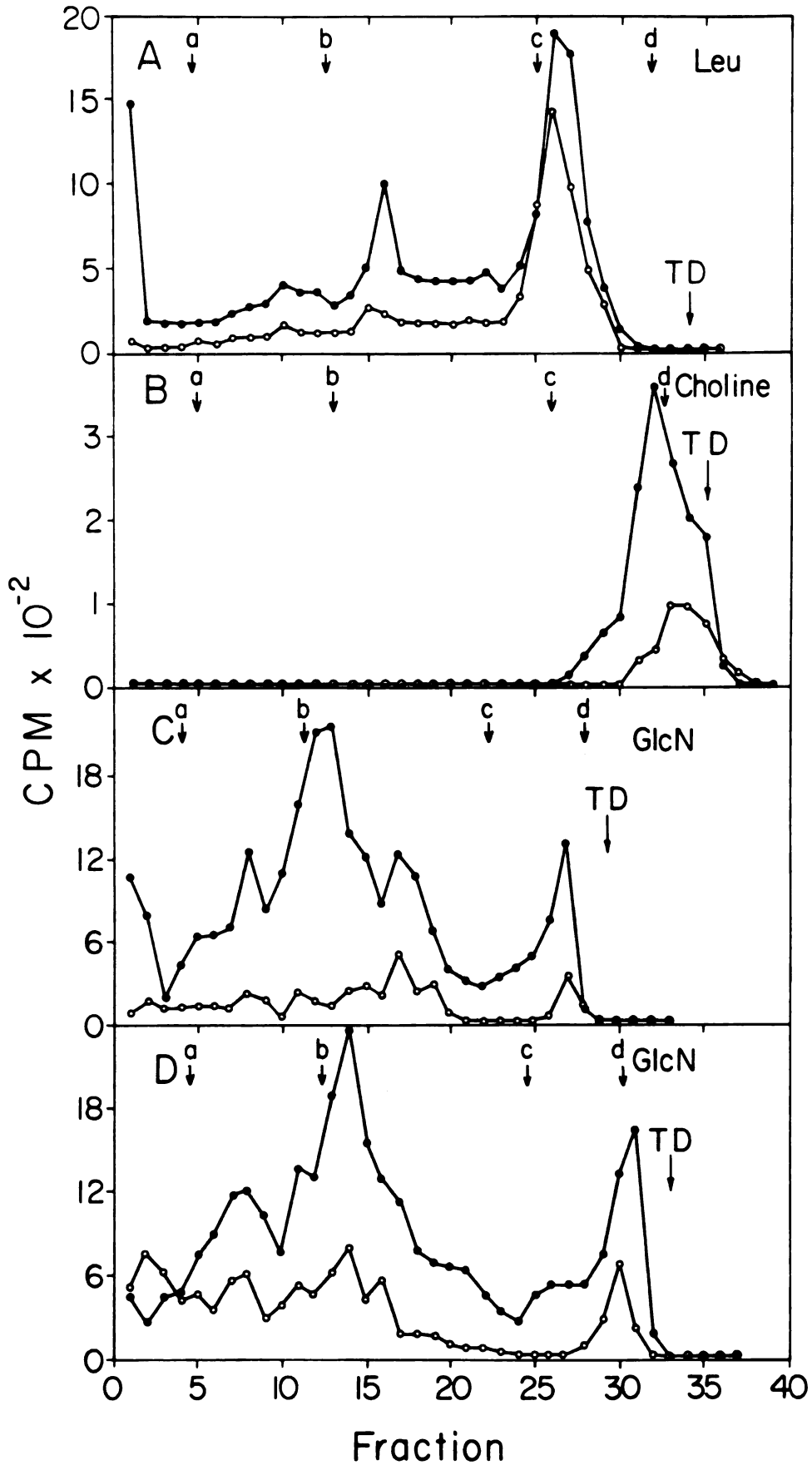

Figure 2

in low molecular weight substances. These data suggest that Thy-1.2 associated complexes are heterogeneous in nature containing proteins, glycoproteins, glycolipids and lipids. The fact that glycolipids and a ^{3}H-leucine and ^{3}H-glucosamine labeled molecule of between 51,000 and 56,000 daltons were consistently precipitated with anti-Thy-1.2 sera suggests that this glycoprotein ($\sim$53,000 daltons) and a glycolipid are candidates for Thy-1.2 antigen.

The Thy-1 antigenic substance contained in the shed complexes was examined by another approach which solubilizes membrane complexes. Peak I samples of culture medium from ^{3}H-leucine and ^{3}H-glucosamine labeled S.49.1 cells were fractionated over a Sephacryl-S-200 column containing 1% deoxycholate (DOC) (Figure 3). ^{3}H-leucine labeled molecules were found primarily in substances of between 67,000 and 200,000 m.w. and reduced levels of radiolabeled molecules were detected in lower molecular weight substances. Shed material labeled with ^{3}H-glucosamine was found primarily in large complexes >200,000 daltons and substances of about 67,000 daltons. Also, some radioactivity was detected in two peaks of low molecular weight in which ^{3}H-G_{M1} ganglioside co-chromatographed with labeled molecules of between 14,000-20,000 m.w. Four groups of pooled fractions, A(19-25), B(26-31), C(32-40) and D(41-60), were extracted by ethanol precipitation to test the isolated glycoproteins and glycolipids for their Thy-1.2 antigenic properties. Glycoproteins and glycolipids extracted from group A (>45,000 m.w.) could induce a significant anti-Thy-1.2 PFC response of 121 plaques. Extracted molecules from groups B, C and D did not induce any detectable PFC responses. This result suggests that a glycoprotein or glycolipid >45,000 m.w. contains the Thy-1.2 antigenic moiety.

Figure 3. Chromatography of Peak I material from ^{3}H-leucine and ^{3}H-glucosamine labeled S.49.1 cells over a 1% deoxycholate Sephacryl-S-200 column. Anti-Thy-1.2 PFC responses induced by pooled fractions are represented by the open bar. The column was calibrated by the following molecular weight standards: BD (blue dextran 2 x 10^{6} daltons); BSA (bovine serum albumin 67,000 daltons); STI (soybean trypsin inhibitor 23,000 daltons); LYS (lysozyme 15,000 daltons); and PR (phenol red 376 daltons).

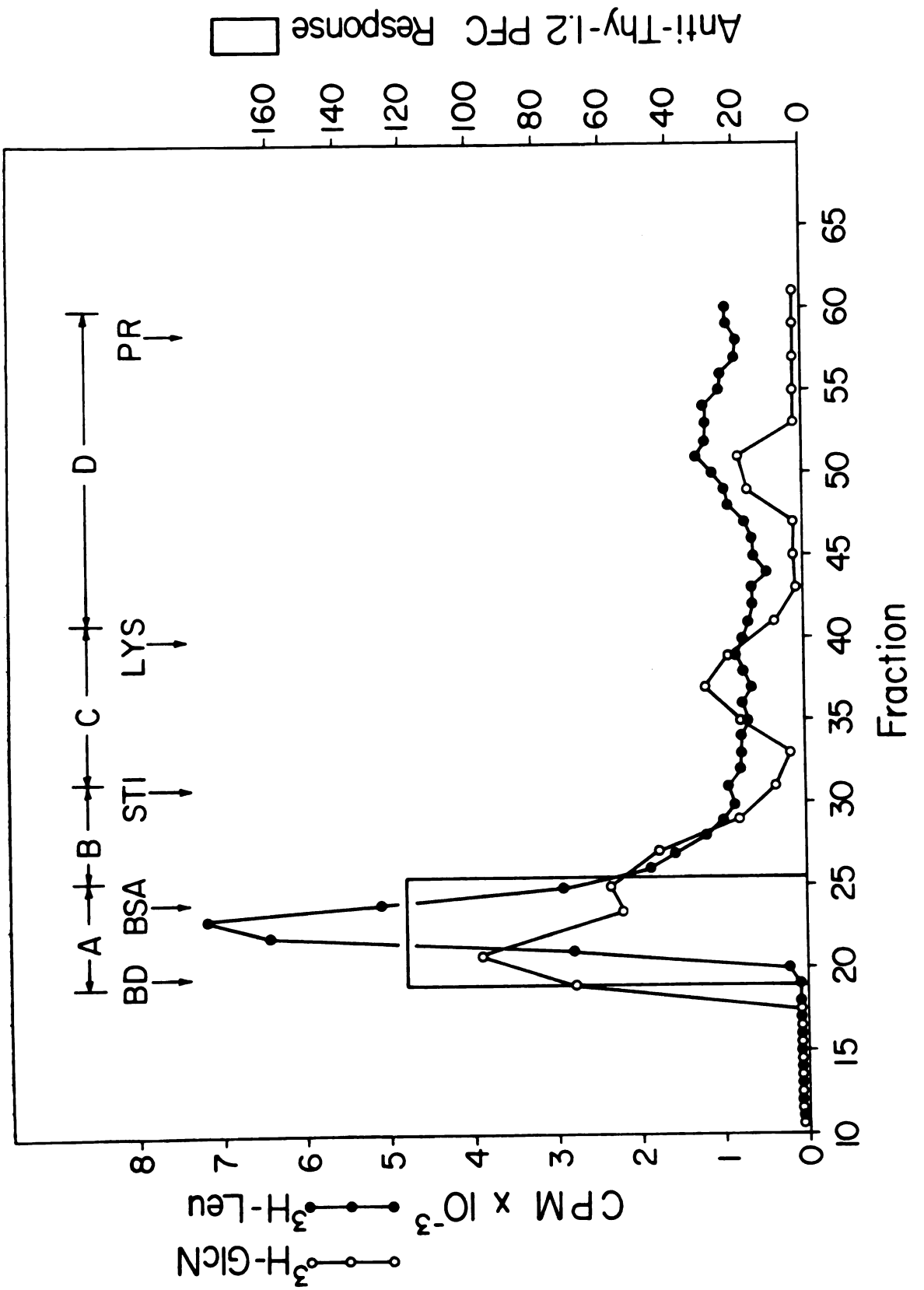
Anti-Thy-1.2 PFC Response
160
140
120
100
80
60
40
20
0
PR
D
LYS
C
STI
B
BSA
A
BD
CPM x 10⁻³
³H-GlcN
³H-Leu
8
7
6
5
4
3
2
1
0
10
15
20
25
30
35
40
45
50
55
60
65
Fraction

Figure 3

Further evidence for the glycolipid nature of Thy-1.2 antigen in shed macromolecules from S.49.1 and BW 5147 cells was provided in experiments using rabbit anti-G_{M1} sera (C3H brain G_{M1}) to adsorb lymphoblastoid culture medium. Varying dilutions of anti-G_{M1} sera were incubated with S.49.1 culture medium for 12-16 hours at 4°C before addition to spleen cell cultures (Table II). Pretreatment of S.49.1 culture medium with anti-G_{M1} sera diluted 1:20 reduced the anti-Thy-1.2 PFC response 58% ($p<0.01$ by Student's t-test), while higher dilutions had no significant effect on the PFC response. In similar experiments, pretreatment of BW 5147 culture medium with anti-G_{M1} (1:15) significantly reduced the anti-Thy-1.1 PFC response by 58% ($p<0.01$), while higher dilution had no effect (Table III). In other experiments (not presented) normal rabbit serum (1:15 to 1:25 dilutions) pretreatment of either S.49.1 or BW 5147 culture medium had a small (10-20% reduction) but consistent abrogating effect on induction of anti-Thy-1 PFC responses. These data suggest that the G_{M1} ganglioside is associated with the released Thy-1 complexes.

Discussion

High molecular weight complexes containing Thy-1 antigen shed from S.49.1 and BW 5147 lymphoblastoid cells were studied to ascertain their composition by a variety of biochemical approaches. Anti-Thy-1 sera immunoprecipitation of culture medium containing shed material radiolabeled for protein, glycoprotein, glycolipid or lipid components demonstrated that the Thy-1 associated complex contained all of these molecules. Column chromatography of these radiolabeled culture media and subsequent precipitation with

Table II

Inhibition of Anti-Thy-1.2 PFC Response by Anti-G_{M1} Sera

Immunizing Medium[a]	Treated[b] with	Dilution of Antiserum	Anti-Thy-1.2 Response PFC/10^7 Cells (CBA Target Cells)
BW5147 (1.1)[c]	---[d]	---	6.3 ± 2.3[e]
S.49.1 (1.2)	---	---	158.0 ± 28.1
S.49.1 (1.2)	anti-G_{M1}	(1:20)	66.0 ± 17.8
S.49.1 (1.2)	anti-G_{M1}	(1:40)	115.0 ± 37.0
S.49.1 (1.2)	anti-G_{M1}	(1:80)	128.0 ± 22.3

[a]AKR (Thy-1.1) spleen cultures with 2 x 10^7 cells in 0.5 ml medium were treated with 0.5 ml of lymphoblastoid culture medium.

[b]Culture medium was pretreated with anti-G_{M1} sera at the respective final concentration for 12-16 hours at 4°C before addition to spleen cultures.

[c]Thy-1 allotype in parentheses.

[d]No additions.

[e]Means ± standard errors of 6 cultures/groups.

Table III

Inhibition of Anti-Thy-1.1 PFC Response by Anti-G_{M1}

Immunizing Medium[a]	Treated[b] with	Dilution of Antiserum	Anti-Thy-1.1 Response PFC/10^7 Cells (AKR Target Cells)
S.49.1 (1.2)[c]	---[d]	---	6.0 ± 0.7[e]
BW 5147 (1.1)	---	---	369.0 ± 31.1
BW5147 (1.1)	anti-G_{M1}	(1:15)	156.0 ± 23.2
BW5147 (1.1)	anti-G_{M1}	(1:40)	327.0 ± 24.3
BW5147 (1.1)	anti-G_{M1}	(1:100)	336.0 ± 30.0

[a]Culture medium added in the same manner as Table II to CBA (Thy-1.2) spleen cells.

[b]Treatment same as Table II.

[c]Thy-1 allotype in parentheses.

[d]No additions.

[e]Means ± standard errors of 6-7 cultures/groups.

anti-Thy-1 sera indicated that all the radiolabeled complexes containing Thy-1 were detected in large macromolecules greater than 2×10^6 daltons (Table I). The fact that each of the radiolabeled precursors examined was incorporated into these large complexes in relatively similar quantities suggested that these substances were important to the integrity of the shed complex, but precluded identification of a radiolabeled Thy-1 antigen.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of radiolabeled Thy-1 associated compounds demonstrated a heterogeneity in its constituents. At least four proteins and/or four glycoproteins were present in the shed complex with significant quantities of phospholipids and glycolipids. This composition of the shed Thy-1 associated complexes is consistent with present concepts of membrane structures (22) and dynamics of membrane turnover (1). Based upon the evidence presented in this report it is proposed that the large molecular weight complexes containing Thy-1 antigen released from S.49.1 and BW 5147 cells are actually membrane fragments containing most of the constituents normally contained in the intact and functional plasma membranes of these lymphoblastoid cells.

The nature of the Thy-1 antigen was studied using partially characterized components as starting material for immunoprecipitation of radiolabeled shed material with anti-Thy-1.2 or anti-Thy-1.1 alloantisera followed by SDS-PAGE analysis of these precipitates. Although anti-Thy-1.1 serum served as a control for non-specific precipitation of radiolabeled material from S.49.1 (Thy-1.2) cells, there has been some previous observation of some cross reaction between these two alloantisera (12). In a few experiments not

reported here, normal mouse serum (NMS) was used. These NMS precipitates yielded somewhat smaller amounts of radiolabeled substances and eliminated most small peaks of radioactivity seen with anti-Thy-1.1 sera.

Radiolabeled (S.49.1) proteins, glycoproteins, glycolipids and lipids were precipitated with anti-Thy-1.2. Comparison of gel profiles from ^{3}H-glucosamine labeled Peak I, plus immune precipitates of whole medium and Peak I material, indicates that anti-Thy-1.2 sera precipitated predominantly high molecular weight material from unfractionated culture medium. These data indicated that a ^{3}H-leucine labeled molecule of 51,000 m.w. and ^{3}H-glucosamine labeled substance of very similar molecular weight were consistently precipitated, suggesting these two molecules were the same, thus being a glycoprotein. Two radiolabeled components were consistently precipitated to a much greater degree with anti-Thy-1.2 sera than with anti-Thy-1.1 sera. These substances were a 53,000 dalton glycoprotein and the low molecular weight glycolipids which co-migrated with the G_{M1} ganglioside marker, and just ahead of the Pyronin-Y tracking dye.

Several researchers have also studied the biochemical composition of Thy-1 antigen extracted from the cell surface of rat and murine thymocytes (8-10) and brain (10) and lymphoblastoid cells (23). The nature of rat Thy-1 molecule found in thymocytes and brain tissue has been characterized as a glycoprotein of 25,000 m.w. and 24,000 m.w., respectively (10). Trowbridge et al. (8) and Arndt et al. (9) studied the glycoprotein properties of Thy-1 in murine thymocytes and demonstrated that molecules of 25,000 daltons were predominant in SDS-PAGE analysis of anti-Thy-1 immunoprecipitates.

Zwerner et al. (23) determined that the Thy-1.1 antigen extracted from the surfaces of BW 5147 cells was a glycoprotein of approximately 25,000 daltons. Vitetta et al. (6) proposed that Thy-1 antigenicity was contained in glycolipids, because Thy-1 antigen in unstimulated thymocytes could not be labeled with ^{3}H-amino acids in a four hour incubation period, while ^{3}H-galactose could label the Thy-1 molecule. Study of lymphoblastoid cell variants (in expressing Thy-1) led Trowbridge et al. (24) to postulate that Thy-1 antigenicity resided in the carbohydrate moiety and Thy-1 variants lacked essential glycosyl transferases to add the essential carbohydrate to the protein backbone. Miller and Esselman have demonstrated that G_{M1} ganglioside can adsorb cytotoxicity of anti-Thy-1 sera, suggesting a ganglioside nature for Thy-1 (12). Anti-Thy-1 precipitates of solubilized membranes from ^{3}H-galactose labeled murine thymocytes were analyzed by SDS-PAGE (3,6). A broad radioactive peak of 35,000 daltons material and radiolabeled substances of 65,000 and 75,000 daltons, plus significant radioactivity detected in low molecular weight substances migrating with glycolipids was observed. In a similar approach with ^{125}I-labeled anti-Thy-1.2 precipitates, Walsh et al. (25) found some radiolabel only at the very top of the gel and the majority of radioactivity at the bottom in the migration area of glycolipids.

Determination of the glycolipid or glycoprotein nature of Thy-1 in shed complexes was pursued by deoxycholate column chromatography of S.49.1 culture medium from ^{3}H-leucine and ^{3}H-glucosamine labeled cells. Radioactivity associated with these labels was primarily found in material greater than 45,000 daltons. Ethanol extractions were performed to remove toxic deoxycholate and

precipitate glycoprotein and glycolipids and exclude proteins (10,26). Only ethanol extracted molecules larger than 45,000 m.w. could induce an anti-Thy-1.2 PFC response, while lower molecular weight substances had no effect, suggesting either a glycoprotein or glycolipid of this size contained Thy-1 antigenicity. In support of these findings were the results from Letarte-Muirhead et al (36) and Arndt et al. (9), who demonstrated that rat or mouse Thy-1 in similar deoxycholate columns had molecular weight of 55,000 daltons. This result for molecular weight is larger than without DOC, due to significant quantities of deoxycholate binding to the Thy-1 molecule and forming micelles, thus increasing its size and molecular weight (27).

Inhibition of anti-Thy-1 PFC responses by anti-G_{M1} ganglioside sera pretreatment of immunizing lymphoblastoid culture medium suggested a glycolipid nature for both Thy-1.1 and Thy-1.2 antigens. The lack of specificity demonstrated by this rabbit antisera directed against C3H (Thy-1.2) G_{M1} brain ganglioside is not unusual. Previous studies showed that both C3H-G_{M1} and AKR-G_{M1} could adsorb anti-Thy-1.2 and anti-G_{M1} (C3H) sera, though greater quantities of AKR-G_{M1} were required (12). Furthermore, rabbit anti-mouse brain associated Thy-1 sera react with both Thy-1 allotypes (28). These results suggest that shed complexes from S.49.1 and BW 5147 cells contain a glycolipid with G_{M1} ganglioside properties. However, recent research utilizing G_{M1} and Thy-1 glycoprotein from murine thymocytes in *in vitro* secondary anti-Thy-1 PFC responses gave interesting observations (29). These experiments indicated that both Thy-1 glycoprotein and G_{M1} glycolipid could equally serve to induce significant anti-Thy-1 PFC responses that retained their Thy-1 allotype specificity. Further analysis of G_{M1} ganglioside with different organic solvents allowed the isolation

of a new glycolipid containing all of the Thy-1 antigenic activity, which migrated, separately and below G_{M1} on thin layer chromatographic plates (29). The biochemical nature of this new Thy-1 glycolipid is still under investigation, though sialic acid residues appear to be essential for antigenicity as prolonged neuraminidase treatment of this glycolipid will alter its antigenicity (29).

In conclusion, comprehension of the exact nature of Thy-1 antigen still has to be determined. We postulate that the Thy-1 antigenicity is carried by a carbohydrate moiety which can be attached to either a protein or lipid backbone as in blood group substances or viral antigens (30,31). Also, from the evidence presented in this communication we postulate that Thy-1 associated complexes shed from S.49.1 and BW 5147 cells are large molecular weight membrane fragments released in the form of liposomes, and the Thy-1 antigenic moiety is present on both a glycolipid and a glycoprotein molecule.

References

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

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

3. 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.

4. Calafat, J., J. Hilgers, W. J. Van Blitterswijk, 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.

5. Lutz, H. V., S.-C. Liu, and J. Palek. 1977. Release of Spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of Spectrin-free vesicles. J. Cell Biol. 73:548.

6. 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.

7. Kucich, V. N., J. C. Bennett, and B. J. Johnson. 1975. The protein nature of Thy-1.2 alloantigen as expressed by the murine lymphoblastoid line S-49.1 TB·2·3. J. Immunol. 115: 626.

8. 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 (Lond.) 256:652.

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

10. Williams, A. F., A. N. Barclay, M. Letarte-Muirhead, and A. J. Morris. 1977. Rat Thy-1 antigens from thymus and brain: their tissue distribution, purification, and chemical composition. Cold Spring Harbor Symp. Quant. Biol. 41:51.

11. Esselman, W. J. and H. C. Miller. 1974. Brain and thymus lipid inhibition of anti-brain-associated θ-cytotoxicity. J. Exp. Med. 139:445.

12. 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.

13. 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.

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

15. Freimuth, W. W., W. J. Esselman, and H. C. Miller. 1978. Release of Thy-1.2 and Thy-1.1 from lymphoblastoid cells: partial characterization and antigenicity of shed material. J. Immunol. 120:(in press).

16. 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 responses. Cell. Immunol. (submitted).

17. Porzio, M. A. and A. M. Pearson. 1977. Improved resolution of myofibrillar proteins with sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Biochim. Biophys. Acta 490:27.

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

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

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

21. Van Eijk, R. V. W. and P. F. Mühlradt. 1977. Carbohydrate incorporation in plasma membranes of mouse thymocytes stimulated by concanavalin A. Eur. J. Biochem. 78:41.

22. Singer, S. J. 1974. Molecular biology of cellular membranes with applications to immunology. Adv. in Immunol. 19:1.

23. Zwerner, R. K., P. A. Barstad and R. T. Acton. 1977. Isolation and characterization of murine cell surface components. I. Purification of milligram quantities of Thy-1.1. J. Exp. Med. 146:986.

24. Trowbridge, I. S. and R. Hyman. 1975. Thy-1 variants of mouse lymphomas: biochemical characterization of the genetic defect. Cell 6:279.

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

26. 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. 143:51.

27. Kuchel, P. W., D. G. Campbell, A. N. Barclay, and A. F. Williams. 1978. Molecular weights of the Thy-1 glycoproteins from rat thymus and brain in the presence and absence of deoxycholate. Biochem. J. 169:411.

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

29. 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. (submitted).

30. Pigman, W. 1977. Blood group glycoproteins. In *The Glycoconjugates*. Edited by M. I. Horwitz and W. Pigman. Academic Press, New York, p. 611.

31. Burns, W. H. and A. C. Allison. 1975. Virus infections and immune responses they elicit. In *The Antigens* vol. 3. Edited by M. Sela. Academic Press, New York, p. 479.