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# MEMBRANE EXPRESSION OF THY-1.2 AND $G_{M1}$ GANGLIOSIDE ON DIFFERENTIATING T LYMPHOCYTES

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ABSTRACT

MEMBRANE EXPRESSION OF THY-1.2 AND $G_{M1}$ GANGLIOSIDE ON DIFFERENTIATING T LYMPHOCYTES

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C3H mouse bone marrow cells were separated by discontinuous BSA gradient centrifugation. Marrow cells from the 17-19%, 19-21%, 21-23%, 23-25%, 25-27% interfaces and the cell pellet were treated with thymic factor (TF) or with *Vibrio cholerae* neuraminidase (VCN), followed by anti-Thy-1.2 and anti-$G_{M1}$ ganglioside antisera. Antigens determined by anti-Thy-1.2 and anti-$G_{M1}$ cytotoxicity responses were expressed either with TF or VCN within a thirty minute incubation. Cells expressing these antigens after VCN or TF treatment were concentrated in the 19-21% layer and the 21-23% layer whereas there was little or no antiserum cytotoxicity in the other layers. A small amount of Thy-1.2 or $G_{M1}$ could be detected by cytotoxicity on 19-21% layer marrow cells within fifteen minutes of either TF or VCN treatment. Treatment of 19-21% layer cells with TF or VCN had no effect on anti-H-2 cytotoxicity. Pretreatment of gradient purified TF-treated marrow cells with cholera toxin or choleragenoid (which bind cell surface $G_{M1}$) abrogated the cytotoxicity of anti-Thy-1.2 or

anti-$G_{M1}$ without affecting anti-H-2 cytotoxicity. Pretreatment of Thy-1.2 positive C3H thymocytes with cholera toxin or choleragenoid greatly reduced the cytotoxicity of anti-Thy-1.2 antisera without affecting the cytotoxicity of anti-H-2 antisera. Nude mouse splenocytes, after treatment with VCN or TF, were susceptible to the lytic action of anti-Thy-1.2 and anti-$G_{M1}$. The possibility that nonspecific autologous antibodies were responsible for anti-Thy-1.2 cytotoxicity toward VCN-treated marrow cells was eliminated because anti-Thy-1.2 was not cytotoxic for VCN-treated AKR (Thy-1.1) marrow cells.

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# LITERATURE REVIEW

## I. *Thy-1 Antigen*

A. *Definition and Distribution.* In 1964, Reif and Allen (1) demonstrated that thymocytes and peripheral T lymphocytes and central nervous tissue of mice shared allogeneic antigenic determinants. This antigen system occurs in two allelic forms resulting in two alloantigens, Thy-1.1 (θ - AKR) and Thy-1.2 (θ - C3H). These antigens were at first thought to be mouse strain specific, however, recent studies show that cross-reacting or identical antigenic determinants to the Thy-1 antigen exist on thymocyte or brain of all rat tissue (2).

Thy-1 is a membrane-bound antigen found in large amounts only on thymocytes and brain of mice and rats (1). It is found in smaller amounts on peripheral thymus-derived cells, fibroblasts of mouse or rat (3), epidermal cells (4) and cells in the peripheral and central nervous system (5). It is not found on bone marrow or bone marrow-derived lymphocytes (1), plasma cells (6), granulocytes, macrophages, or red blood cells (7).

B. *Isolation.* Because of the membrane-bound nature of the Thy-1 antigen, different attempts at solubilization and purification of the molecular complex have resulted in variable isolation products. The three most widely used isolation techniques concerning the Thy-1 molecule are detergent solubilization (8), immunoprecipitation (9), and enzyme solubilization (10).

1. *Detergent solubilization.* Letarte-Muirhead et al. (11) characterized a molecule isolated from rat thymocytes by detergents, including Lubrol-PX or deoxycholate. In high concentrations this molecule could inhibit the cytotoxic activity of Balb/c anti-Wista rat brain antibodies to rat thymocytes. This "solubilized" Thy-1 antigen was said to contain 25-50% of the original antigenic activity of intact cells or membrane. Use of the solubilized undiluted antigen to attempt inhibition of C3H anti-AKR antisera resulted in a weak but definite inhibition of cytotoxicity. Using anti-rat brain antisera to measure activity, the antigen isolate was characterized by sucrose gradient centrifugation and gel filtration. The molecular weight of the anti-Thy-1 antiserum-reactive isolate was estimated to be 28,000. A high frictional coefficient in sucrose gradient suggested that the Thy-1 molecule was asymetric, possibly containing a hydrophobic tail.

Chemical characterization of the Thy-1 molecule isolated by Barclay and Letarte-Muirhead (12) and defined by reactivity with anti-rat Thy-1 antisera showed that the Thy-1 molecules from both thymocytes and brain are glycoprotein of 25,000 molecular weight. It was estimated that 30% of brain or thymocyte Thy-1 is carbohydrate but that the carbohydrate composition isolated from respective tissues is not the same. It was suggested that the antigenicity of the molecule resides in the protein moiety. In support of this hypothesis was the finding by Barclay that the antigenicity of the molecule was destroyed by pronase treatment (13). However, it was also found that the antigenicity was not significantly decreased by other proteolytic enzymes, including trypsin and papain. This may suggest that the

protein may be only partially responsible for antigenicity or may be a carrier for the carbohydrate and/or other undefined antigenic units.

Further studies by this group of investigators using other cell lines including a Thy-1 positive tumor (14) and using highly concentrated and purified glycoprotein illustrated the reproducibility of the isolation procedure on other Thy-1 bearing tissues. However, the Thy-1 activity isolated was oftentimes less than 25% of the originally estimated cytotoxic values. These findings and those concerning pronase digestion of the isolated antigen may suggest a bias in the selection procedure used for isolation of the Thy-1 molecule and that all components of the molecule essential for specificity and optimum antigenicity within the cell membrane were not isolated by these procedures.

In 1966 Reif and Allen (5) demonstrated that the θ - C3H (Thy-1.2) antigen of thymocytes was non-dialyzable and sensitive to lipid solvents, e.g. chloroform and methanol. Noting these findings, Esselman and Miller (15), attempted extraction of this molecule from mouse thymocytes and brain by lipid solvents. In association with auxilliary lipids, the antigen could inhibit the cytotoxicity of rabbit anti-mouse brain antisera (anti-BAθ). This anti-BAθ inhibitory substance was located in the upper, water soluble phase of a Folch partition, and co-migrated by thin-layer chromatography with the ganglioside $G_{D1b}$. Moreover, it was found that isolated mouse brain $G_{D1b}$ ganglioside could also inhibit the cytotoxicity of anti-BAθ. These investigators proposed that the antigenic determinant of the Thy-1 antigen of mouse brain may be associated with glycolipid material.

Esselman and Miller later demonstrated the specific inhibitory capacity of isolated AKR and C3H gangliosides for anti-Thy-1 sera. As in other antigen systems, cross-reactivity was also observed at higher levels of antigen concentration (16). Esselman and Kato (17) demonstrated the ability of pentasaccharides derived from AKR and C3H gangliosides to inhibit specifically their respective antisera in a hapten inhibition assay. Cross-reaction was again observed when allo-antigen was used in higher concentrations (e.g. AKR oligosaccharide with anti-Thy-1.2).

2. *Immunoprecipitation.* Vitetta et al. (18) used immunoprecipitation methods with specific anti-Thy-1.1 antiserum to murine thymocytes to attempt solubilization of the Thy-1 molecule. In contrast to Letarte-Muirhead, it was found that deoxycholate destroyed the antigenic activity of the Thy-1 molecule. However, the estimate of a molecular weight of 35,000 for Thy-1 was in reasonable agreement with Letarte-Muirhead.

Vitetta et al. found that $^{125}$I-labeled Thy-1 or $^{3}$H-galactose-labeled Thy-1 was released from cultured thymocytes (19). The amount of Thy-1 found in the culture medium was proportional to the amount of time the cells were cultured. Based on the ability to label the antigen using lactoperoxidase, and on finding molecules shed in the culture medium, Vitetta et al. proposed that the Thy-1 molecule is peripherally located in the cell membrane. This release of soluble fragments from the membrane of T cells has been suggested by other investigators as a possible mechanism for T cell-mediated cytotoxicity and stimulation of B cells (20, 21).

Characterization of the molecule immunoprecipitated by Vitetta et al. demonstrated an NP-40 labile substance of lower density than protein which, in cell culture, could not be labeled with $^{3}H$-labeled amino acids. Based on these findings, it was proposed that Thy-1 may contain a glycolipid portion in its antigenic unit (19). Atwell et al. (22) utilized the lactoperoxidase method of radioiodination of cell surface protein followed by immunoprecipitation to obtain a non-dissociable anti-Thy-1 inhibitory molecule. Mobility on gel electrophoresis in sodium dodecylsulfate (SDS) with reference molecules resulted in a molecular weight estimate of 60,000. Because of the stability of the molecule in SDS, the authors suggested that although the molecular weight is higher than previous estimates, it is probably not a molecular conglomerate. It was suggested that the detergents used by other investigators to extract Thy-1 from membrane may cause dissociation of the antigenic complex.

3. *Enzyme Solubilization.* Kuchich et al. (10) utilized a limit papain digest of the Thy-1.2 positive S49 murine cell line. Limited papain digestion of S49 cells resulted in solubilization of material containing a molecular complex capable of inhibiting the cytotoxicity of anti-Thy-1.2 antisera against S49 cells. Further increased exposure of S49 cells to pronase digestion reduced the amount of Thy-1.2 activity, indicating further degradation of the molecule. Protease digestion of the papain-solubilized material also resulted in a decrease in antigenicity. The authors suggested that a protein in the released Thy-1 molecule is essential for its antigenicity.

Later studies suggested that sialic acid participates in the antigenicity of the molecule (23). Using papain-solubilized digests of the anti-Thy-1 inhibitory molecule, it was found that neuraminidase destroyed this inhibitory activity. Sialic acid in isolated form was also shown to be an effective cytotoxic inhibitor. The authors then suggested that not only is the Thy-1 molecule glycoprotein in character but that it may also contain lipid material

In contrast to the data of Kuchich et al., Schlesinger and Gottesfeld (24), found the effect of neuraminidase on Thy-1 antigenicity to be minimal. However, in addition to different concentrations of enzyme and antiserum used by the two groups of investigators, it should be noted that Kuchich et al. used papain-solubilized antigen from S49 Thy-1 positive tumor cells as a substrate for neuraminidase, while Schlesinger utilized whole thymocytes as substrate.

This summary of findings on the nature of the Thy-1 molecule illustrates that the nature of the antigen is still undetermined. The antigen has, at various times, been proposed to be of low molecular weight, high molecular weight, glycoprotein, glycolipid, or a carbohydrate in combination with both lipid or protein. These differences in findings may indicate the complexity in structure of the Thy-1 molecular complex on the cell and illustrate the controversy in utilizing different isolation techniques to define chemically a cellular antigen.

## II. *Thymic Factor*

A. *Introduction.* Evidence for a humoral factor from the thymus has been based on the restoration of immune function to thymectomized

mice either by injection of thymic extracts or by insertion of thymus grafts encapsulated in cell-impermeable Millipore chambers (25). Evidence for immune restoration was seen in the ability of the animal subject to produce antibodies to sheep red blood cells (26) as well as to reject skin allografts (27). Bach et al. (28) attempted characterization of an *in vitro* model of thymic extract restoration of immunocompetence. It was postulated that the capacity of lymphoid cells to form rosettes correlated with restoration of immunocompetence and the ability of the cells to react with an antigen.

B. *Isolation of a thymic inducer substance.* Komuro and Boyse (29) demonstrated the effect of an extracted thymic inducer substance on the expression of known T cell antigens on murine spleen and bone marrow cells. The distribution of some of these antigens is shown as follows:

| ANTIGEN | DETECTED IN |
|---|---|
| Thy-1 (θ) | T lymphocytes, skin, brain (1) |
| TL | T lymphocytes in thymus only (30) |
| Ly-1, Ly-2, Ly-3 | T lymphocytes (31) |
| MSLA | Thymocytes and other T cells (32) |
| $G_{IX}$ | Thymocytes of certain mouse strains (33) |

Using Thy-1.2 antigen as a marker for thymic factor inducer function, it was found that a significant proportion (about 20%) of those bone marrow or spleen cells settling on the 10-30% interface of a bovine serum albumin (BSA) discontinuous gradient could be induced to express the Thy-1 antigen. Although not tested with murine marrow

cells, Komuro and Boyse also demonstrated the ability of the thymic extracts to induce an increase in the expression of TL antigen on splenocytes (29).

Scheid et al. (34) further studied the ability of a substance called "thymosin" to induce T cell differentiation. Thymosin, purified by the method of A. Goldstein (35), had also been employed in the work of Komuro and Boyse. This substance was shown to induce the expression of TL, Thy-1, $G_{IX}$, Ly-1, Ly-2, and Ly-3 antigens on pre-differentiated cells of mouse bone marrow. It was suggested by Scheid that perhaps this thymic humoral substance may have a mechanism of action similar to other isolated hormones, that is, thymosin may function via an adenylate cyclase system, and that cyclic AMP may be a participant in the initiation of cellular differentiation. It was shown that use of aminophylline, a phosphodiesterase inhibitor, or insulin, a purported adenylate cyclase inhibitor, was reported to cause a decrease in the ability of thymosin to cause expression of Thy-1 antigen on marrow cells. Scheid then suggested that any agent able to induce or interfere with the production of cyclic AMP may alter the ability of thymosin to induce antigen expression. It was reasoned that cyclic AMP may act as a non-specific trigger of a pre-Thy-1 positive cell to convert to a Thy-1 positive cell.

The thymic factor-induced rise in cyclic AMP was illustrated by several investigators. Kook and Trainin (36) utilized a substance isolated from calf thymus termed THF (thymic humoral factor). They confirmed an establishment of immunocompetence (graft vs. host reaction) with a concommitant rise in cyclic AMP after injection of THF. Bach

et al. reported that cyclic AMP ($10^{-9}$) could be substituted for circulating thymic factor *in vivo* to cause rosette forming cell production in adult thymic-irradiated mice. The effect of thymic factor and cyclic AMP were found to be synergistic when co-incubated with spleen cells (37).

Several apparently non-specific substances were then found by Scheid et al. (38) to be capable of inducing the appearance of Thy-1 antigen on marrow cells from the 10-23% albumin layers of a BSA discontinuous gradient. All products could also cause increased cyclic AMP levels. These substances included epinephrine, propranolol, isoproterenol, endotoxin, poly A:U, and even calf muscle (38). Bach et al. found that the prostaglandins $PGE_1$ and $PGE_2$ could also increase cyclic AMP levels and mimick the effect of thymic factor, and that this effect could be inhibited by indomethacin, an inhibitor of prostaglandin synthetase (39). However, it was discovered that some of these factors, including endotoxin, propranolol, and calf muscle were also found to induce B cell differentiation, as measured by an increase in complement receptor (C 3 receptor) expression (29).

G. Goldstein isolated a product from the thymus which he named thymopoietin (TP)(40), a substance which was originally used for study on its neuromuscular effects in the disease myasthenia gravis. It was shown that this product was specific for T cell differentiation. During the isolation of TP, a second polypeptide was isolated. This polypeptide, named ubiquitin (UBIP) was found to be present in several tissues and was able to induce both T and B cell differentiation *in vitro*. Pre-incubation of marrow cells with propranolol inhibited the

induction of Thy-1 antigen on pre-T cells or complement (C 3 receptor) receptors on the surfaces of B cells. It was then suggested that UBIP may be a non-specific inducer of Thy-1 expression, and its presence in several tissues may explain why non-thymic sources may cause antigen expression (41).

C. *Intracellular events of TF induction.* Storrie et al.(42) studied the intracellular effect of thymopoietin during thymocyte differentiation. Induction of both TL and Thy-1 antigen expression by thymopoietin was blocked by actinomycin D, camptothecin, or cordycepin; inhibitors of DNA replication did not block induction. The authors concluded that the induction of Thy-1 and TL antigen expression may require DNA transcription and RNA translation but not DNA replication. This was in support of earlier work by Komuro and Boyse (29) indicating that thymic factor induction could be inhibited with cycloheximide.

However, Rotter and Trainin (43) found a 50-100% increase in the uptake of thymidine using phytohemagglutinin (PHA) or concanavalin A (con A) as mitogens for spleen cells preincubated with THF, a purported T cell specific substance. Basch and Goldstein, using thymopoietin (44) observed no measurable effect of spleen cells to con A or PHA. Using bone marrow cells, previously shown by TP induction of Thy-1 antigen to contain precursor T cells, Goldstein observed no thymidine uptake in response to con A or PHA incubation (4). This discrepancy in findings may have been a consequence of different purification procedures of the thymic extracts used by the two investigators.

D. *Isolation and characterization of TF.* Schlesinger et al. (46) have been able to synthesize partially the amino acid sequence of thymopoietin as isolated and determined by G. Goldstein (47). This peptide, corresponding to a portion of the sequenced natural polypeptide displayed a similar selectivity of action for differentiating T cells, although only displaying 3% of the activity of whole isolated thymopoietin.

E. *The target cell for thymic factor.* Till and McCullough (48) originally proposed the existence of a pluripotential stem cell of bone marrow. Using sublethally irradiated mice, they demonstrated recolonization of spleen by cell clones of injected bone marrow. These colony forming units (CFUs) were shown to be capable of differentiating into granulocytic or lymphocytic cell lines. It was suggested that perhaps this pluripotential stem cell of marrow was the target cell for thymic factors, that it migrated to the thymus and was triggered to differentiate into a lymphoid cell.

Studies of marrow cell subpopulations using density gradient methods delineated at least two more cell subsets. One subset is a relatively small population of proposed "re-circulating" T cells, demonstrated as possessing the Thy-1 antigen (49), capable of initiating graft vs host responses (30), and able to respond to PHA (51). El-Arini and Osoba (52) located a much larger population of marrow cells which they termed "progenitor T cells". This population was weakly susceptible to the cytotoxic activity of anti-Thy-1 serum, and gave rise to immunocompetent cells in the spleens of irradiated isogeneic recipients within 15 days of transplantation. After isopycnic centrifugation, this cell was shown to be slightly denser than a CFU (stem) cell

(1.050-1.059 gm/cm$^3$) and lighter than a bone marrow Thy-1 positive cell (1.069 gm/cm$^3$). The density of this cell was estimated at 1.063 gm/cm$^3$. The presence of this weakly Thy-1 positive cell was also demonstrated by Loor and Roelants (53). Work by Turner et al. (54) illustrated that cells of stem cell and pro-thymocyte densities (1.050 and 1.063 gm/cm$^3$, respectively) fall within the 21-23% BSA layers of a discontinuous gradient. Thus those cell populations shown by Boyse, Sheid, and Goldstein to be inducible by various thymic extracts contained both pro-thymocytes and stem cells and the target cell for the thymic factor preparation was undefined.

Indirect evidence suggests that the thymic factor-inducible cell from bone marrow is committed to thymocyte differentiation. The ability of pro-thymocytes to be induced by various substances (thymic factors, cAMP, poly A:U, endotoxin) is suggested by Boyse as a non-specific response of pre-programmed cells to become Thy-1 positive (55). In addition, slightly Thy-1 positive cells can be detected in nude mice (mice congenitally lacking a thymus, termed nu/nu)(56) and approximately 35% of nu/nu marrow cells from the 10-23% interface of a BSA gradient can be induced to become Thy-1 positive after thymic factor incubation. It was reasoned that the thymus is not essential for differentiation of stem cells, that the cells susceptible to thymic factors are "pre-programmed" to become T cells (29). The thymic factor target cells may not be stem cells and may instead be progenitor T cells. It is not possible to eliminate the suggestion that these "pro-thymocytes" may instead be "pro-immunocytes" (57), able to be triggered into functional T or B cells depending on their environment. This may explain the existence of cells with both B and T cell markers. The target cell(s)

for thymic factor has not been defined. The step-wise differentiation from marrow stem cell to pre- and post-thymic lymphocytes to peripheral T lymphocytes may display a differential sensitivity of these cells to thymic factors.

III. *Neuraminidase*

A. *Introduction.* Treatment of cells with *Vibrio cholerae* neuraminidase (VCN) results in the removal of 2-3 and 2-6 glycosidically-linked sialic acid from mucopolysaccharides, glycoprotein and glycolipids on the cell surface (58,59,60). This enzyme treatment of the cell causes less negative charge on the cell surface (61). Several alterations can be induced as a result of this neuraminidase treatment. These include rendering the cell more easily phagocytosed (62), enhancing monocyte phagocytic activity (63), inhibition of viral hemagglutination (64), and changing homing characteristics of injected cells (65). The results of these experiments on neuraminidase treatment of cells suggested that cells may possess the genetic information for antigens not displayed phenotypically. Neuraminidase will alter the antigenicity of several cell lines, including erythrocytes, marrow cells, lymphocytes, and tumor cells.

B. *Neuraminidase treatment of erythrocytes.* Sialic acid is an indigenous component of the cells of many different animal species (66, 67). Currie and Bagshawe (68) originally proposed that some antigens on the cell surface may be masked by a "sialo-mucin coat". Treatment of erythrocytes with neuraminidase releases greater than 90% of the sialic acid (69). Barth and Singla (70) noted a differential effect of VCN treatment on the antigenicity of sheep, horse, chicken, and goat red blood cells. Neuraminidase treatment significantly reduced the

antigenicity of both sheep and horse red blood cells, but did not effect goat or chicken erythrocytes. The authors proposed that this differential effect may function in determining inter-species variations in antigenicities. This is supported by the finding that autologous transfusion of neuraminidase-treated erythrocytes results in their rapid removal from the circulation (69). The use of $^{51}Cr$-labeled cells illustrated their removal by the liver and spleen (71).

The effect of neuraminidase-treated red blood cells on cell surface interactions was studied by Schlesinger and Galili (72). Neuraminidase treatment of sheep red blood cells resulted in the formation of stable (non-dissociable) rosettes with peripheral T lymphocytes or thymocytes. The authors proposed that the formation of stable rosettes is probably due to a decrease in charge separation between the two cells or to exposure of new cross-linkage determinants.

C. *Neuraminidase treatment of lymphocytes.* Several groups of investigators have studied the effect of neruaminidase treatment on the exposure of molecules on lymphocyte membranes. Schlesinger and Amos (73) found an increase in guinea pig serum cytotoxicity on neuraminidase-treated C57BL (Thy-1.2 positive) murine thymus cells. A similar effect was observed using neuraminidase-treated spleen cells. An eightfold increase was observed in the effect of anti-Thy-1.2 antiserum on these cell populations. In addition, up to 30% of bone marrow cells became Thy-1.2 positive as defined by cytotoxicity. The amount of TL antigen on thymocytes increased after neuraminidase treatment, but there was no measurable increase on marrow cells. This illustrated a possible differential effect of the enzyme on antigen

exposure. From these experiments Schlesinger proposed that neuraminic acid may cover the Thy-1 molecule in its natural state and inhibit its expression and/or detection. It was concluded that neuraminic acid is probably not a part of the antigenic determinant(s) of the Thy-1 molecule, nor is it an antigenic component of the guinea pig serum-detectable surface molecule. It was suggested that the TL antigen may not be "hidden" by sialic acid.

Barton and Rosenberg (74) arrived at a somewhat different explanation of the effect of neuraminidase on lymphocyte cell surfaces. It was suggested that only the more peripheral sialyl compounds are susceptible to neuraminidase treatment. The sialolipids, e.g., may be more deeply embedded in the cell membrane and may not be susceptible to the action of VCN.

Reisner supported the suggestions of Schlesinger that neuraminidase may be exposing "hidden" membrane components to the antisera (75). The similar reactivity of non-treated thymus cells and VCN-treated peripheral lymphocytes was a result of the increased amount of sialic acid released from peripheral cells. Neuraminidase-treated thymocytes release significantly less neuraminic acid than other lymphoid cells.

Other authors confirmed the increased cytotoxic effects of a variety of antisera to neuraminidase-treated lymphocytes. Rosenberg and Rogentine (76) showed that normal human sera contained lytic antibodies directed against autologous human lymphocytes that had been pre-treated with VCN. The sera used were highly concentrated. These investigators later found that pre-incubation of B-D galactosyl residues with normal human sera abrogated the cytotoxicity of the sera to

autologous VCN-treated lymphocytes (77). Within the HL-A system, the specific antigen reactions were found to be unchanged after VCN treatment. In some cases, however, an increase in the weaker antigen reactions or the appearance of a new familial antigen (78) was observed. The original work of Schlesinger and Amos using heteroantisera to VCN-treated lymphoid cells was tested in other systems. Schlesinger and Gottesfield described naturally occurring antibodies in guinea pig serum directed against neuraminidase-treated murine cells (79). Rosenberg et al. discovered a similar toxicity of normal rabbit antisera to neuraminidase-exposed determinants on human cells (80). This heterospecific cytotoxicity can be absorbed with untreated mouse or human tissue (81). It has therefore been suggested that a non-specific diffusely occurring antigen may be unmasked in addition to those specific antigens unmasked after neuraminidase treatment, e.g. as in the case of the "T antigen" which is revealed on red blood cells after neuraminidase treatment. This phenomenon was originally demonstrated in 1947 by Burnet and Anderson (82), who noted the spontaneous agglutination of human red blood cells after VCN treatment.

Recently it has been suggested that it is not the exposure of "cryptic" antigens that increases antiserum cytotoxicity after neuraminidase treatment, rather that other factors play a significant role. A possible explanation for increased serum cytotoxicity was increased cell fragility. However, enzyme-treated cells were shown not to be more fragile and therefore more susceptible to antiserum treatment. For example, human lymphocytes were shown to give negative cytotoxicity reactions with many antisera to the human HL-A system, survive well in

tissue culture, and respond as well to lectin stimulation as untreated cells (78).

Since the fixation of complement by IgG antibody may depend on the attachment of two molecules (83), it has been proposed that neuraminidase treatment may make the simultaneous attachment more feasible by exposing additional complement receptors. Complement activation would then be more probable. It was also suggested that a site essential for completion of the complement pathway may be further uncovered with neuraminidase treatment (84). Because of this proposal some investigators state that VCN-treated cells use more complement and therefore appear more sensitive to antisera (84). Others suggest that perhaps the complement is used more efficiently (75). It was suggested by these authors that absorption studies would be helpful in clarifying the controversy of exposure of cryptic antigens. Ray and Simmons (85) found no difference in absorptive capacity of anti-H-2 antisera with VCN-treated lymphocytes.

Some authors have proposed that these naturally occurring antibodies are directed against neuraminidase that has absorbed to the surface of the lymphocyte (86). Rosenberg and Rogentine found that heat-inactivated neuraminidase did not increase cellular cytotoxicity, nor did neuraminidase treatment of cells in the presence of sialic acid. Moreover, in absorption experiments using large numbers of cells untreated with enzyme, some cytotoxic activity could be removed (76). It has been suggested that heat inactivation may not be an appropriate control for testing neuraminidase adherence to cells, as the active site may serve in cellular attachment (87).

D. *Neuraminidase and tumor cells.* In 1964, Kirby et al. (88) proposed that the pregnant female fails to reject the fetal trophoblast as an allograft because of the presence of a protective coat. This masking of the trophoblast cells protects the placental cells of fetal origin from interaction with and rejection by maternal lymphocytes. Trypsinization and subsequent *in vitro* culture of trophoblast cells allows for gross cytolysis by allogeneic or maternal lymphocytes. A fibrinoid coat was said to be removed with trypsinization, thus unveiling the masked antigens. This fibrinoid coat was later shown to be an amorphous sialomucin (89). These findings led to a theory proposed by Currie and Bagshaw that cancer cells maintain a "protective coat" which inhibits cytotoxic reactivity with the immune system of the host. This coat was proposed to be composed of "pericellular sialomucins" (68).

Histochemical studies of tumor cells have illustrated that many tumor cell lines contain a high content of acid mucopolysaccharides (90,91,92). Moreover, Defendi and Gasic observed a significant increase in surface sialomucin of hamster embryo cells undergoing malignant transformation by polyoma virus (93).

As with erythrocytes and lymphocytes, the treatment of tumor cells with neuraminidase resulted in increased lysis of the cells in heteroantisera (94). Neuraminidase treatment also increased the ability of lymphoid cells or fibroblasts to stimulate allogeneic or syngeneic lymphocytes in the one-way mixed lymphocyte culture (95,96). Several investigators have demonstrated increased immunoreactivity and decreased growth of neuraminidase-treated tumor inocula (97,98,99).

Hughes et al. demonstrated that this increased immunoreactivity to the tumor after injection of VCN-treated cells could be abrogated by concomitant injection of anti-Thy-1 antiserum (94).

Simmons et al. demonstrated the induction of immunity to a tumor in a syngeneic system. An established methylcholanthrene-induced fibrosarcoma could be ablated by the injection of neuraminidase-treated cells of the same tumor. Injection of enzyme-treated cells of other tumors would not result in the disappearance of the fibrosarcoma, thus suggesting the specificity of the treatment (100). The possible application of this system to clinical use has been discussed (101).

E. *Cellular neuraminidases.* The finding of several mammalian cellular neuraminidases complicates the previous studies using exogenous enzyme and simultaneously supports the theory that neuraminidases may play a role in cell membrane dynamics. Carabelli (102) originally demonstrated the presence of neuraminidase in liver, brain, spleen, and intestine of the rat. It was later characterized in tumor cells (103) erythrocytes, and leukocytes (104).

Neuraminidase activity has been estimated in several areas of the cell, including microsomes (103), mitochondria (105), and in the soluble fraction of tissue homogenates (106). Studies using rat brain illustrated the presence of at least two distinct cellular neuraminidases, one lysosome-derived and one soluble (106). The enzymes display different pH optima and ion sensitivities and perhaps different substrate specificities. Gielin suggests that soluble neuraminidases prefer low molecular weight substrates, e.g. neuraminlactose, whereas high molecular weight glycoproteins or glycolipids (in gangliosides) are preferred

by lysosomal neuraminidases (107). This suggestion is supported by the findings of Schengrund et al. who found a plasma membrane-marker enzyme associated neuraminidase which was also associated with ganglioside (108). This enzyme is highly active for these membrane-bound gangliosides (109). The specificity for this substrate was suggested by Tettamanti, the enzyme not showing as high affinity for sialoglycoproteins (109). However, Yohe and Rosenberg (110) suggest that sialolipid or sialoglycoprotein are both susceptible to membrane-bound neuraminidase.

It has been suggested that this enzyme may participate with other cell membrane-bound enzymes and susceptible molecules in membrane alteration and/or cell-cell communication (107, 110).

IV. *Cholera Toxin*

A. *Introduction.* Cholera toxin is the specific exotoxin produced by *Vibrio cholerae* which is active on epithelial cells of the small intestine causing acute diarrhea (111). In its purified form the toxin is a protein of molecular weight 84,000, composed of six subunits (112).

B. *Mechanism of Action.* The ability of cholera toxin to stimulate intracellular adenylate cyclase activity was originally investigated using an isolated section of rabbit or dog ileum and measuring the diarrheogenic effect of cyclic AMP levels of intestinal cells. It was found that the toxin was rapidly fixed to these cells (113). Others found that the activity of cholera toxin, as mediated by cyclic AMP could be studied in other systems, including adipocytes (114), liver cells(115), liver plasma membrane (116), adrenal cells (117), and lymphocytes or fibroblasts in tissue culture (118).

Cuatrecasas (119) and Vaughn (120) studied the effect of cholera toxin on the cyclic AMP-mediated lipolytic response of adipocytes. Alloxan, an inhibitor of hormone-stimulated adenylate cyclase activity in cells, would inhibit the lipolytic response caused by cholera toxin. Inhibitors of prostaglandin synthesis, indomethacin and sodium salicylate, did not block this lipolytic effect. In addition, and despite suggestions to the contrary (121), it was found that inhibitors of RNA and protein synthesis, including actinomycin D, cycloheximide, and puromycin were also reported to be ineffective in blocking the lipolytic effect of the toxin (119).

Van Heyningen (114) first noted that the required incubation time for measuring the maximum lipolytic response caused by cholera toxin was not dependent on the presence of the toxin. Washing the toxin from the cells after ten minutes of incubation still resulted in the maximum lipolytic response. Moreover, studies by Cuatrecasas revealed a one hour delay in the lipolytic response (119). It was postulated that the toxin-cell membrane receptor complex must initially undergo alteration until it is active, and that this new active complex stimulated the biologic response, as mediated by adenylate cyclase.

C. *The receptor for cholera toxin.* Cuatrecasas (122) studied the binding activity of $^{125}$I-labeled cholera toxin. At very low concentrations (less than $10^{-10}$M) of cholera toxin, greater than 50% of the toxin bound to liver cell membranes. This could easily be replaced by unlabeled toxin. It was found that the binding of the toxin was directly proportional to the amount of liver cell substrate provided. The same activity of the purified toxin could be found using isolated adipocytes.

It was found that certain glycoproteins could partially inhibit the interaction of $^{125}$I-labeled toxin with hepatocytes. These included fetuin and rat liver membrane glycoprotein in high concentrations. Tests using simple sugars to attempt inhibition of the toxin were not successful at the concentrations tested and in the form used. Some of the sugars tested were D-glucose, D-mannose, D-fucose, D-glucosamine, D-galactosamine, N-acetyl D-glucosamine, N-acetyl D-galactosamine, and N-acetyl neuraminic acid. High concentrations of D-galactose effected a small amount of inhibition. Use of a *Clostridium perfringens* neuraminidase digest of fetuin resulted in a thirty percent increase in inhibitory capacity. Studies employing purified gangliosides showed that they were the most potent inhibitors of $^{125}$I-labeled cholera toxin binding to hepatocytes. Simple glycosphingolipids such as glucose- and galactose-ceramides were not inhibitory. Sequential addition of terminal galactose or N-acetylgalactosamine increased the inhibitory capacity. Adding sialic acid to the galactose of a galactose-glucose-ceramide moiety lead to a minimum of a twenty-fold increase in inhibition of $^{125}$I-labeled cholera toxin binding. $G_{M1}$ ganglioside was found to be the most potent inhibitor and was found to cause inhibition at nanogram concentrations. Addition of N-acetylneuraminic acid to $G_{M1}$ ganglioside decreased inhibition by fifty percent. Neuraminidase digests of $G_{D1a}$ and $G_{T1}$ gangliosides improved their inhibitory abilities. It was stated that the relative order of potency of binding inhibition by gangliosides was: $G_{M1}$ (20 ng/ml), $G_{D1a}$(0.7 μg/ml), $G_{M2}$(1 μg/ml), $G_{T1}$(1.8 μg/ml), $G_{L4}$(17 μg/ml), $G_{M3}$(50 μg/ml) (122). It was also noted that predigestion of the liver cell membranes with neuraminidase or with B-galactosidase

enhanced the binding of $^{125}I$ labeled toxin. Further evidence for the binding of cholera toxin to ganglioside was obtained with membrane extraction studies. Toxin binding to hepatocyte membranes was greatly decreased by pre-treatment of the membranes with choloroform-methanol solvents by methods known to be effective in extracting gangliosides. Moreover, material in the water soluble phase of these extracts were inhibitive of toxin binding to untreated hepatocyte membranes (122).

From these data it was suggested by Cuatrecasas that the natural receptor for cholera toxin may be a ganglioside-containing material. Further evidence to support this hypothesis was the finding that exogenous gangliosides, especially $G_{M1}$ ganglioside, is a potent inhibitor of cholera toxin binding to cells and that pre-incubation of $G_{M1}$ ganglioside with hepatocytes increases binding and biological activity of toxin. This was confirmed in reports by Pierce and by Holmgren et al. (123, 124). Hollenberg et al. (125) later supported this finding by utilizing an SV40-transformed cell line which lacks chemically detectable $G_{M2}$, $G_{M1}$, and $G_{D1a}$ gangliosides, and which bound $< 10\%$ of $^{125}I$-labeled toxin.

D. *Choleragenoid.* Finkelstein and LoSpalluto, while attempting to purify the active cholera toxin (choleragen) also purified a biologically inactive derivative of the choleragen molecule with a molecular weight of 58,000. The two molecules are antigenically identical. The derivative now called "choleragenoid" is said to lack the biologically active subunit of the molecule (111).

Cuatrecasas found that the affinity of the $^{125}I$-choleragenoid molecule for the liver cell membrane was identical to that of the

active toxin. Binding studies showed that isolated gangliosides, pre-incubated with the choleragenoid, would inhibit the biologically inactive toxin from binding to the cell membrane, and that pre-incubation of hepatocytes with ganglioside caused enhanced binding of the labeled molecule to the cells. Thus this biologically inactive substance retained binding properties virtually identical to that of the active cholera enterotoxin. Choleragenoid is specific in its ability to inhibit the lipolytic effect of subsequently added cholera toxin to adipocytes. It is not able to inhibit the lipolytic effect of epinephrine or glucagon (126).

Choleragen is, therefore, proposed to be composed of two subunits, a 58,000 dalton "binding" subunit, which reacts with cell membranes, and another 38,000 dalton "active" subunit, which is responsible for its biological activity (127).

E. *Interaction of cholera toxin with the cell membrane.* Bennett reported that the choleragen molecule forms a detergent-stable complex with adenylate cyclase (128). The $^{125}$I-labeled cholengen is found to be associated with adenylate cyclase activity when activated by the molecule. It was proposed that it is unlikely that the molecule enters the eytoplasm in soluble form, as < 1% of the $^{125}$I-labeled toxin could be found in the cytoplasm (129).

Craig and Cuatrecasas (130) used fluorescein-labeled cholera toxin to demonstrate temperature-dependent changes in cell membrane fluidity of rat lymph node cells on cholera toxin binding. The membrane-bound cholera toxin, when warmed from 0°C to 37°C for 20-30 minutes changed from a diffuse surface fluorescence to a polar fluorescence (capping).

This capping phenomenon was partially inhibitable by colchicine (60%), Con A (80%), $NaN_3$ (96%), and NaF (78%). Antibody to the fluorescent-labeled cholera toxin inhibited the capping. Because the capping phenomenon was demonstrable and because it was inhibitable by anti-cholera toxin antibodies it was suggested that cholera toxin may be at least a bivalent molecule. This suggestion was supported by the finding that $G_{M1}$-bound agarose beads will bind holera toxin, to which lymphocytes will then bind.

Cuatrecasas proposed that the membrane complex of cholera toxin and its probable ganglioside receptor undergoes formation of an inert toxin-ganglioside complex which is converted to an active state after an approximate twenty to thirty minute lag phase. This active unit then undergoes lateral diffusion within the membrane until it reacts with adenylate cyclase to form a complex. The adenylate cyclase is thus activated (131).

In support of this proposed mechanism of action are the following findings:

1) The extreme persistence of the biological effects of cholera toxin (132).

2) The irreversible binding of $^{125}I$-labeled cholera toxin to cell membranes (119).

3) Suggestions that the toxin may become incorporated into membrane phospholipid (133).

4) The hydrophobic properties of the active subunit of the toxin (134).

The lag period is then the time required for the inactive ganglioside-cholera toxin complex to laterally diffuse to, react with, and activate adenylate cyclase. This suggestion is in accord with the

proposed mechanisms of action of other hormones in regards to membrane fluidity (120).

Gill and King (135) propose that the activation of adenylate cyclase by cholera toxin is not through a one-to-one association of the toxin with cyclase, but through an NAD-dependent enzymatic reaction. They demonstrated that intact choleragen or its active subunit can catalyse the hydrolysis of NAD to ADP-ribose and nicotinamide. As previously demonstrated with diptheria toxin (136), the active subunit of cholera toxin is proposed to mediate NAD-dependent ADP-ribosylation of an acceptor, in this case L-arginine methyl ester or a related amino acid residue, as contained in a protein. This acceptor protein may be adenylate cyclase or an associated molecule (137).

Flores (138) demonstrated that the addition of NAD to toxin-hepatocyte incubation mixtures increased the activity of adenylate cyclase twofold. The addition of NAD eliminated the latent period required to demonstrate adenylate cyclase activity in the experiments by Cuatrecasas. Further support for the possible NAD-mediated activation of adenylate cyclase was the finding that cholera toxin inhibits protein synthesis in mouse spleen cells as measured by $^{3}$H-uridine incorporation (139). This finding was analogous to information previously obtained on diptheria toxin. It is known that diptheria toxin causes inhibition of protein synthesis as a result of NAD-dependent ADP-ribosylation of the protein receptor molecule "elongation factor II(EFII)" (140).

F. *Cholera toxin as a marker for lymphoid cell populations.* The work by Cuatrecasas and others has demonstrated the high affinity

of cholera toxin for specific ganglioside material. Cholera toxin was not found to bind strongly to oligosaccharides in the concentrations used, although the inability of cholera toxin to bind to cell membrane glycoprotein has not been excluded (141).

Studies by Esselman and Miller implicated a possible antigenic similarity between the undefined Thy-1 molecule and $G_{M1}$ ganglioside (16). In possible correlation with this finding is the study by Stein-Douglas (142) showing the value of employing anti-ganglioside antisera in detecting thymus-derived (Thy-1 positive) cell populations.

Sultzer and Craig (139) demonstrated the ability of cholera toxin to inhibit specific mitogen stimulation on T cells. This indicated that cholera toxin receptors may be found on T cell populations. However, in much higher concentrations, cholera toxin was shown to inhibit the effect of a specific B cell mitogen, thus indicating a lower affinity of the toxin for this cell type. The studies by Sultzer and Craig may indicate a high affinity receptor for cholera toxin on T cells. The receptor may be glycolipid and/or glycoprotein in composition and may be associated with the Thy-1 molecule. The possible ability of cholera toxin to bind to B cells may indicate a similar receptor for the toxin on this cell population which may exist in lower concentrations or in a less available form.

## INTRODUCTION TO EXPERIMENTAL REPORT

Enzyme treatment of intact cells has been used a a means of releasing exposed labile components and of exposing hidden or cryptic determinants and receptors. Treatment of lymphocytes and erythrocytes with *Vibrio cholerae* neuraminidase (VCN) has been reported to expose an antigen (or antigens) which can be detected by human or animal sera (76,77,81,143,144,145). Some of these workers (143,145) reported a VCN exposed antigen determined by autologous antisera which was shared by erthyrocytes and all types of leucocytes. Schlesinger (24) reported that the treatment of mouse lymphoid cells with VCN resulted in the increase of a guinea pig serum cytotoxicity receptor but did not change the amounts of exposed H-2 antigen or Thy-1 antigen. The cytotoxicity of normal guinea pig serum for VCN-treated murine tumor cells was apparently the result of exposure of glycopeptides containing terminal B-galactosyl residues (94).

*Vibrio cholerae* neuraminidase does not hydrolyze sialic acid from cell surface gangliosides even though some gangliosides are VCN-labile after isolation (74). The gangliosides are apparently deeply localized in the intact surface of some cell types and are sterically inaccessible to VCN. The sialic acid of $G_{M1}$ ganglioside is not hydrolyzed by VCN even in isolated form (74). Hakomori (146,147) has shown that antiserum

to a glycolipid found in fetal red cell membrane did not cause hemolysis even though the glycolipid was externally exposed (i.e. it may be radiolabeled using specific cell surface labeling techniques). Treatment of these cells with neuraminidase, however, expresses the glycolipid antigen and renders the cells susceptible to anti-glycolipid antisera. Glycolipids may be deeply localized in the intact membrane where they may be sterically inaccessible to VCN or to antibodies. $G_{M1}$ ganglioside has been identified as the cell surface receptor for cholera toxin (148,149). Cholera toxin is composed of a subunit which imparts biological activity and subunits (called choleragenoid) which bind the toxin to the cell surface by binding $G_{M1}$ (150).

It has previously been demonstrated that Thy-1 antigen is exposed on marrow stem cells after one hour of treatment with thymic factor (151). The finding that C3H brain $G_{M1}$ ganglioside was capable of inhibiting the cytotoxicity of anti-Thy-1.2 antiserum led to the proposal that $G_{M1}$ ganglioside is structurally related to the Thy-1 antigen (15,16,152). In view of the rapid appearance of Thy-1 antigen on marrow stem cells after thymic factor treatment it is suggested that Thy-1 may be expressed on marrow cells by either thymic factor or *Vibrio cholerae* neuraminidase treatment and that these antigens may be blocked by treatment with cholera toxin or choleragenoid.

## MATERIALS AND METHODS

*Cells.* Bone marrow cells or thymocytes were harvested from C3H/ST or C3H/HeJ mice at age 4-7 weeks (Jackson Laboratories, Bar Harbour, Maine). Nude mice (nu/nu) on a BALB/c background were a generous gift of Dr. M. Pienkowski of Michigan State University. Thymuses were trimmed of all nodal tissue and were gently teased with forcepts in Eagle's medium to free cells from connective tissue. The thymocytes were then disaggregated using 18 and 25 gauge needles. An average of $1 \times 10^8$ nucleated cells were obtained per thymus. Bone marrow cells were harvested from the femurs and tibias using a 27 gauge needle and were suspended in Eagle's medium. Eight to ten mice would normally yield $1 \times 10^7$ nucleated cells with 85% vilability. These cells were then subjected to centrifugation in a discontinuous gradient of bovine serum albumin utilizing layers of 19,21,23,25,27 and 35% BSA. The BSA was prepared from a 35% stock solution (65 ml tris buffer and 35 g of dialyzed and lyophilized BSA, Grade V, Sigma Chemical Co.) according to the method of Dicke, et al (153). The NaCl concentration of the buffer was adjusted such that the osmolarity of 17% BSA was 310 mOsmols. After centrifugation, distinct cell layers were observed at the interfaces. Cells removed from the respective interfaces had the following composition as observed by

light microscopy. The 17-19% BSA interface contained dead cells in low numbers and cellular debris. The 19-21% BSA interface contained primarily mononuclear cells of 99% vilability as did the 21-23% BSA interface (although the latter also contained some polymorphonuclear cells). The 25-35% BSA interface contained a diverse population of 99% viable marrow cells, including erythrocytes. The pellet contained predominantly erythrocytes and debris. All cell layers were found to contain only 1 to 3% Thy-1.2 positive cells when tested by cytotoxicity with anti-Thy-1.2 antiserum and complement. Cells in the interfaces of the 19-21% and 21-23% BSA layers were used for experimentation either as a pooled suspension or as separate populations.

*Thymic factor.* Thymic factor (TF), Fraction 3, was prepared from calf thymus according to A. Goldstein, et al. (35). Thymic factor (Fraction 3) used in initial experiments was a generous gift of Dr. Allyn Rule. Thymic factor was also prepared according to the protocol of G. Goldstein (40) and was used as a mixture containing thymopoietin I and II, as well as ubiquitin. Fifty µg of TF was added to $10^6$ cells in a final volume of 1.1 ml and incubated at 37°C for 60 minutes.

*Antisera and complement.* AKR anti-C3H (anti-Thy-1.2) antisera was prepared according to Reif and Allen (1) or obtained from Litton-Bionetics, Inc. Kensington, Md. (Lots 231-55-1 and 231-55-2). Anti-$G_{M1}$ antisera was prepared by multiple injections of Dutch Belt rabbits with isolated C3H brain $G_{M1}$ ganglioside using Freund's complete adjuvant and BSA essentially according to Naikai, et al. (154). The antisera were thermally inactivated at 56°C for thirty minutes and adsorbed with C3H erythrocytes, hepatocytes, marrow cells and agarose.

Specificity of the antisera for $G_{M1}$ was determined by complement fixation. Anti-H-2 antisera, containing antibodies to H-2 specificities 11 and 23 directed against K and I regions of $H\text{-}2^k$, was a gift of Drs. Chella S. David and Donald C Shreffler. Guinea pig complement (Grand Island Biological Co., Grand Island, N.Y.) was adsorbed with agarose. The cytotoxicity of the antisera with added complement was determined by trypan blue exclusion. Marrow cells or thymocytes ($10^6$ cells in 0.1 ml Eagle's medium) were first treated with 0.05 ml of heat inactivated antisera for thirty minutes; 0.05 ml of adsorbed guinea pig complement (diluted 1:3) was then added and the final mixture incubated for thirty minutes at 37°C. Trypan blue (0.05 ml) was added and cells were counted in an eosinophil counter.

*Cholera toxin.* Initial assays involving use of cholera toxin were with a purified filtrate of the toxin supplied by the Cholera Advisory Committee of the NIH, prepared from the procedure of R.A. Finkelstein (155). Later experiments were conducted using a purified cholera toxin filtrate obtained from Schwarz-Mann & Co. (Orangeburg, N.Y.) made according to the preparation protocol of Finkelstein. Choleragenoid was the generous gift of R.A. Finkelstein (150). Unless otherwise noted, 0.1 mg of cholera toxin or choleragenoid was added to $10^6$ cells in a total volume of 1.1 ml. The cells and toxin were then washed twice by centrifugation in Eagle's medium before adding antisera.

*Neuraminidase.* *Vibrio cholerae* neuraminidase was purchased from Koch-Light Laboratories (Burkinghampshire, England, 500 units/ml). Marrow cells ($10^6$ml) of either the 19-21 or 21-23% fraction of BSA in Eagle's medium were treated with 25 units of VCN for 45 minutes at 37°C.

Cells were then washed twice from medium by centrifugation. An aliquot of the active enzyme preparation was inactivated by heating to 70°C for thirty minutes in order to test for nonspecific antigen induction. The specificity of neuraminidase was determined by incubation with $G_{D1a}$ and $G_{M1}$ gangliosides. $G_{D1a}$ ganglioside was found to be completely hydrolyzed to $G_{M1}$ ganglioside and no activity was observed with $G_{M1}$ ganglioside. Neuraminidase activity was also confirmed by reaction with neuraminlactose. No neuraminidase activity was determined in the cholera toxin preparation using $G_{D1a}$ ganglioside as substrate.

*Absorption of anti-Thy-1.* Neuraminidase-treated C3H marrow cells ($5 \times 10^6$/0.15 ml) were added to 0.05 ml of undiluted anti-Thy-1.2 serum and incubated at 4°C for two hours. Cells were centrifuged from the serum and serial dilutions of the supernatant were tested for residual cytotoxicity for C3H thymocytes. An equivalent number of untreated marrow cells were used for absorption as a control.

# RESULTS

*Neuraminidase and thymic factor induced expression of Thy-1.2 and $G_{M1}$.* Bone marrow cells, collected from the interfaces of discontinuous BSA gradients, were washed twice and treated with thymic factor or neuraminidase. Treated cells were washed twice and the appearance of Thy-1.2 and $G_{M1}$ antigens was assessed by cytotoxicity after treatment with antisera and complement. The greatest amount of conversion of TF or of neuraminidase treated stem cells to Thy-1.2 positive cells was observed in the 19-21% interfaces of BSA (Table I). A significant number of Thy-1.2 positive cells was also observed in the 21-23% interface. Similarly, cells demonstrating the greatest cytotoxicity after anti-$G_{M1}$ treatment were in the 19-21% interface. Cells in the 21-23% interface were also slightly positive with respect to $G_{M1}$. Little or no conversion to Thy-1.2 or $G_{M1}$ positive cells was observed for cells in other BSA layers after treatment with thymic factor or neuraminidase.

Neuraminidase treated marrow cells of the 19-21% layer were subjected to lysis by anti-Thy-1.2 antisera prepared in our laboratory or to commercially prepared anti-Thy-1.2 ascites fluid (Table II). These sources of anti-Thy-1.2 antibodies showed similar activity.

Treatment with neuraminidase followed by either normal mouse serum or by rabbit anti-BSA antisera did not result in increased killing of marrow cells. Minimal cytotoxic action of these antisera was observed with non-enzyme treated cells. Marrow cells incubated with neuraminidase in the presence of its specific inhibitor, sialic acid, showed minimal killing when subsequently incubated with antiserum (Table II). A similar product inhibition of the enzyme was observed in experiments involving anti-$G_{M1}$ antiserum. These results indicate that the changes observed are due to the specific enzymatic action of neuraminidase. To insure that the increase in antigenicity was not due to a non-specific mitogenic effect of neuraminidase, the enzyme was inactivated by heat tratment and subsequently incubated with stem cells. Antisera were then added and no significant cytotoxicity was observed above complement controls (Table II). Neuraminidase treatment under the conditions of the assay did not increase the lysis of cells with anti-H-2 antisera and complement (Table II). These data indicated that VCN treatment did not change the quantity of lytic complement receptors, or create additional antigens on the cell surface specified by anti-H-2 sera.

The rate of conversion of marrow cells was estimated by treating cells for various times with thymic factor or neuraminidase (Fig. 1). Figure 1-a, illustrates that the increase in cytotoxicity of anti-Thy-1.2 or anti-$G_{M1}$ ganglioside antisera was proportional to the length of incubation of thymic factor with the cells. Under the conditions of the assay some anti-Thy-1.2 and anti-$G_{M1}$ cytotoxicity appeared after only 15 minutes of incubation. This cytotoxicity

TABLE I

BSA GRADIENT DISTRIBUTION OF BONE MARROW CELLS

SENSITIVE TO THYMIC FACTOR OR NEURAMINIDASE

| BSA Layer Interface | PERCENT DEAD[a] | | | |
|---|---|---|---|---|
| | Thymic Factor[b] | | Neuraminidase[c] | |
| | Anti-Thy-1.2 | Anti-$G_{M1}$ | Anti-Thy-1.2 | Anti-$G_{M1}$ |
| 17-19% | 0 | 2 | 0 | 0 |
| 19-21% | 20 | 32 | 29 | 34 |
| 21-23% | 10 | 5 | 9 | 4 |
| 23-25% | 0 | 5 | 0 | 0 |
| 25-27% | 0 | 6 | 0 | 0 |
| Pellet | 0 | 0 | 0 | 0 |

[a] Cytotoxicity given as percent dead is corrected for complement controls which were usually between 5% and 10% dead. The average results of three experiments are shown. There was less than 15% difference between individual experiments.

[b] C3H cells ($10^6$) were incubated for 45 min at 37°C with thymic factor (50 μg) in a total volume of 1.1 ml. Cells were washed twice after treatment.

[c] C3H cells ($10^6$) were incubated for 30 min at 37°C with 25 units of *Vibrio cholerae* neuraminidase in a total volume of 1.1 ml. Cells were washed twice after treatment.

TABLE II

TREATMENT OF BONE MARROW CELLS WITH NEURAMINIDASE

| NEURAMINIDASE TREATMENT[a] | ANTISERA | PERCENT DEAD MARROW CELLS |
|---|---|---|
| + | Anti-Thy-1.2[b] | 34(31-37)[d] |
| + | Anti-Thy-1.2[c] | 26(21-29) |
| + | Normal Mouse Serum | 2(0-3) |
| +(Sial.)[e] | Anti-Thy-1.2 | 5(0-9) |
| - | Anti-Thy-1.2 | (0-3) |
| +(70°C)[f] | Anti-Thy-1.2 | 3(0-3) |
| + | Anti-$G_{M1}$ | 30(26-34) |
| - | Anti-$G_{M1}$ | 4(2-6) |
| + | Anti-BSA | 1(0-3) |
| +(Sial.)[e] | Anti-$G_{M1}$ | 5(3-6) |
| + | Anti-H-2 | 40(35-45) |
| - | Anti-H-2 | 42(38-43) |

[a] C3H bone marrow cells from the 19-21% BSA layer were incubated with VCN using conditions described in Table I.

[b] Anti-Thy-1.2 serum prepared in our laboratory (see Materials and Methods).

[c] Anti-Thy-1.2 ascites fluid obtained from Litton Bionetics.

[d] Data given is corrected for complement control and is the average of at least three experiments with range in parenthesis.

[e] Sialic acid (100 µg) was added to incubation buffer in a final volume of 1.1 ml.

[f] Neuraminidase was heated at 70°C for 30 min.

reached higher levels, however, after 30 minutes of incubation. Exposure of cells to neuraminidase also resulted in a significant increase in cytotoxicity of the antisera within 15 minutes of treatment (Fig. 1-b). Similar maximum levels of antisera cytotoxicity was obtained for either neuraminidase or thymic factor treated cells. Anti-BSA antisera showed minimal cytotoxicity over the period of the assay. Cell death in untreated controls or in controls containing complement only generally increased from less than 5% at 15 minutes between 10% and 20% over the 60 minute period.

It has been reported that Thy-1.2 antigen could be induced to appear on nude mouse splenocytes utilizing thymic factor (156). The action of neuraminidase on these cells was determined as shown in Table III. Neuraminidase increased the anti-Thy-1.2 susceptibility of nude mouse splenocytes from a background level of 3% to maximum of 10%. This increase was comparable to the increase induced by thymic factor. Furthermore, the cytotoxicity of anti-$G_{M1}$ ganglioside antiserum was also increased from background level of 0% to a high of 48%. This increase was comparable to the increase induced in nude mouse splenocytes with thymic factor (Table III).

The allotypic specificity of anti-Thy-1.2 antisera was demonstrated because of reports that neuraminidase treatment of cells exposes a nonspecific antigen found on many cell types (see discussion). AKR (Thy-1.1) bone marrow cells of the 19-21% layer were treated with neuraminidase in parallel with C3H cells and then incubated with anti-Thy-1.2 antisera and complement (Table IV). Anti-Thy-1.2 antisera was not cytotoxic for VCN treated AKR marrow cells whereas it was cytotoxic

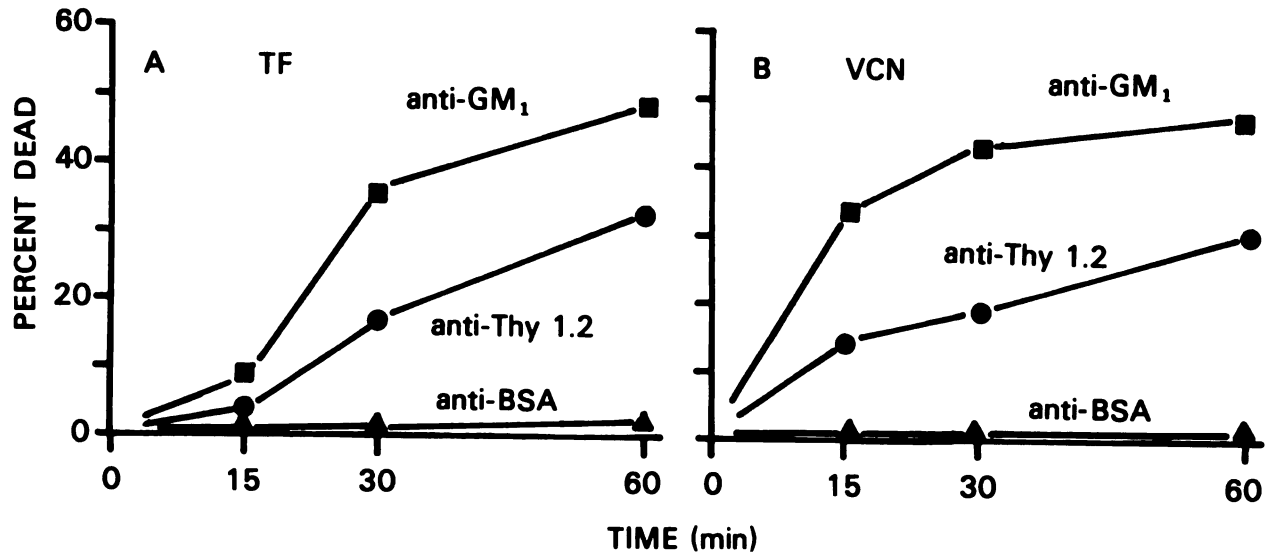


*Figure 1.* Rate of antigen appearance on thymic factor or neuraminidase-treated marrow cells. After incubation with thymic factor or neuraminidase marrow cells were washed and treated with anti-Thy-1.2 (1/40) , anti-$G_{M1}$ (1/40) , or anti-BSA (1/20) antisera. Data shown are the average of two experiments corrected for complement killing.

TABLE III

NEURAMINIDASE OR THYMIC FACTOR TREATMENT OF NUDE MOUSE SPLENOCYTES

| TREATMENT[a] | ANTISERUM | PERCENT DEAD |
|---|---|---|
| None | Anti-Thy-1,2 | 3 |
| Neuraminidase | Anti-Thy-1.2 | 10 |
| Thymic Factor | Anti-Thy-1.2 | 12 |
| None | Anti-$G_{M1}$ | 0 |
| Neuraminidase | Anti-$G_{M1}$ | 48 |
| Thymic Factor | Anti-$G_{M1}$ | 35 |

[a] Neuraminidase and thymic factor were added to $10^6$ splenocytes using conditions described in Table I except that 75 units of VCN were used. Data given is the average of two determinations.

TABLE IV

THY-1.2 ANTISERUM SPECIFICITY FOR C3H MARROW CELLS

AFTER NEURAMINIDASE TREATMENT

| Neuraminidase[a] | Sera | PERCENT DEAD[b] | |
|---|---|---|---|
| | | AKR | C3H |
| + | NMS | 0 | 0 |
| + | Anti-Thy-1.2 | 0 | 17 |
| - | NMS | 0 | 0 |
| - | Anti-Thy-1.2 | 9 | 0 |

[a] VCN was used with marrow cells of the 19-21% interface as described in Table I.

[b] Average data from three experiments. The range of percent dead was less than 15%.

for VCN treated C3H cells. This finding supports the conclusion that Thy-1.2 antigen was exposed by neuraminidase treatment of C3H marrow cells. Neuraminidase-treated marrow cells were used to absorb anti-Thy-1 sera to demonstrate an increase of Thy-1 antigen (Fig. 2). Starting with a dilution of serum which gave approximately 50% cytotoxicity for thymocytes, the neuraminidase-treated marrow cells serially decreased the cytotoxicity in relation to untreated marrow cells.

*Blocking of Thy-1.2 and $G_{M1}$ by cholera toxin.* After incubation of pooled cells from the 19-21% and the 21-23% BSA interfaces with thymic factor, approximately 20% of the population became Thy-1.2 positive as measured by cytotoxicity. If cells treated with thymic factor were washed and subsequently treated with cholera toxin, which binds cell surface $G_{M1}$ ganglioside (148,149) an abrogation in the cytotoxic effect of anti-Thy-1.2 was observed (Table V). The sequence of treatment of cells with thymic factor and cholera toxin was important to inhibit cytotoxic action. Treatment of marrow cells with thymic factor and subsequent incubation of cells with cholera toxin blocks the ability of anti-Thy-1.2 antisera and complement to kill cells. Treatment of cells in the reverse order (cholera toxin, then thymic factor) does not cause a decrease in antiserum cytotoxicity (Table V). Choleragenoid, which is the $G_{M1}$ binding subunit of cholera toxin (150) also exhibited the same ability to block the cytotoxicity of anti-Thy-1.2 antisera.

The ability of cholera toxin to inhibit the cytotoxic effect of specific antisera with cells was also illustrated using anti-$G_{M1}$ ganglioside antisera (Table V). Treatment of TF-treated marrow cells

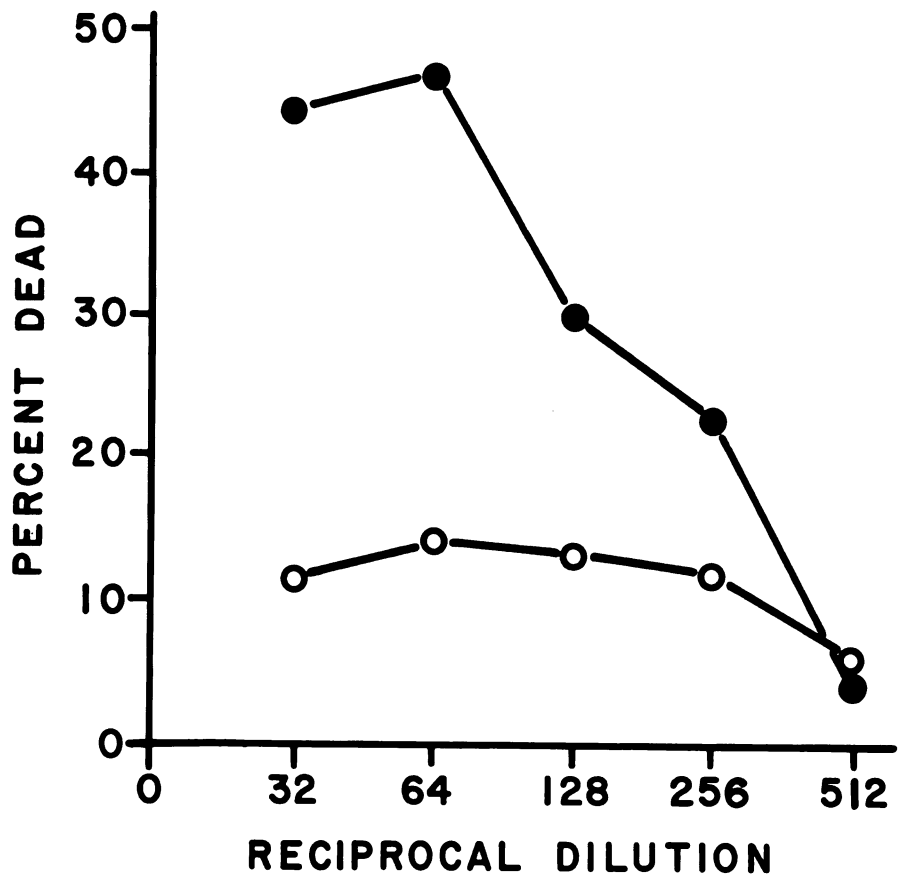


*Figure 2.* Absorption of anti-Thy-1 sera with *Vibrio cholerae* neuraminidase-treated marrow cells. C3H marrow cells used for adsorption were obtained from the 21 and 23% BSA layer. Anti-Thy-1.2 serum was adsorbed with untreated marrow cells or with marrow cells treated with neuraminidase . Each antiserum was titered against thymocytes starting at a dilution which would give 50% cytotoxicity.

TABLE V

INHIBITION OF CYTOTOXIC ACTION OF ANTISERA AFTER TREATMENT OF THY-1.2 POSITIVE CELLS WITH CHOLERA TOXIN OR CHOLERAGENOID

| Sequence of Treatment[a] | | Antisera | PERCENT DEAD MARROW CELLS[b] EXPERIMENT I | EXPERIMENT II |
|---|---|---|---|---|
| TF | - | Anti-Thy-1.2 | 19 | 22 |
| TF | CT | Anti-Thy-1.2 | 0 | 3 |
| - | CT | Anti-Thy-1.2 | 0 | 2 |
| CT | TF | Anti-Thy-1.2 | 18 | 20 |
| TF | CG | Anti-Thy-1.2 | 2 | 3 |
| TF | - | Anti-$G_{M1}$ | 18 | 18 |
| TF | CT | Anti-$G_{M1}$ | 0 | 1 |
| - | CT | Anti-$G_{M1}$ | 0 | 2 |
| TF | - | Anti-BSA | 0 | 0 |
| TF | CT | Anti-BSA | 0 | 0 |
| - | - | Anti-H-2 | 38 | 42 |
| CT | - | Anti-H-2 | 35 | 40 |
| CG | - | Anti-H-2 | 34 | 39 |

[a] Conditions of thymic factor treatment is given in Table I. Cholera toxin or choleragenoid treatment (0.1 mg/$10^6$ cells in 1.1 ml total volume) was performed for 15 min at 37°C. Cells were washed twice after treatment.

[b] C3H bone marrow cells of the 19-21% and 21-23% BSA interfaces were pooled for these experiments. The average of duplicate analysis for each experiment is given.

with either cholera toxin or choleragenoid resulted in a blocking of the cytotoxicity of anti-$G_{M1}$ sera. Anti-BSA antisera did not have a cytotoxic effect after treatment of marrow cells with thymic factor. Further, the inhibitory effect by cholera toxin or choleragenoid was not observed with anti-H-2 antisera, thus illustrating the specificity of the treatment.

The blocking of anti-Thy-1.2 antiserum by pretreatment of Thy-1.2 positive cells with cholera toxin was further studied with C3H thymocytes. The cytotoxic effect of anti-Thy-1.2 antiserum decreased after pretreatment of thymocytes with cholera toxin (Table VI). The blocking, which was observed at several dilutions of antiserum, resulted in a diminiution of cytotoxicity of 36% to 48% at antiserum dilution of 1/100, and at antiserum dilution of 1/200 the diminutions varied from 22% to 30%. As with marrow cells, the cytotoxic action of anti-H-2 antisera on thymocytes was not inhibited by cholera toxin.

TABLE VI

DECREASE IN THE CYTOTOXIC EFFECT OF ANTI-THY-1.2 ANTISERA AFTER TREATMENT OF THYMOCYTES WITH CHOLERA TOXIN

| Cholera Toxin[a] | Anti-Thy-1.2 | PERCENT DEAD THYMOCYTES EXPERIMENT I | EXPERIMENT II | EXPERIMENT III |
|---|---|---|---|---|
| + | 1/100 | 37[b] | 27 | 19 |
| | | (36) | (48) | (37) |
| - | 1/100 | 73 | 75 | 56 |
| + | 1/200 | 9 | 37 | -[c] |
| | | (30) | (22) | |
| - | 1/200 | 39 | 59 | |
| + | NMS(1/20) | 0 | 0 | - |
| | | ( 2) | ( 0) | |
| - | NMS(1/20) | 2 | 0 | |
| + | Anti-H-2 | 85 | - | - |
| - | Anti-H-2 | 84 | | |

[a] Cholera toxin (100 µg) was incubated with $10^6$ C3H thymocytes at 37°C for 30 min. Cells were washed and anti-Thy-1.2 serum and complement were added at the dilutions shown.

[b] Percent of cells killed by anti-Thy-1.2 treatment, value in parenthesis is the decreased observed.

[c] Not determined.

# DISCUSSION

The appearance of T-cell differentiation antigens on marrow or splenic stem cells has been observed by treatment of these cells with various substances including thymic factors, epinephrine, propranolol, and cyclic AMP (34,38,156). Although the usual incubation time for antigen induction has varied from one to two hours it was found that Thy-1.2 antigen expression begins after as little as fifteen minutes of thymic factor treatment (Fig. 1.). The cytotoxicity of anti-Thy-1.2 antiserum increases to one half the maximal level after thirty minutes to reach essentially maximal cytotoxicity after one hour of treatment with thymic factor. Furthermore, the similar appearance of antigens reactive with anti-$G_{M1}$ antisera was observed. It has been previously proposed that $G_{M1}$ ganglioside is identical to or cross-reactive with Thy-1 antigen (15,16,152).

The appearance of Thy-1.2 or $G_{M1}$ on TF-treated marrow cells could be due to: 1. *de novo* biosynthesis of antigen, 2. modification of existing compounds through anabolic or catabolic reactions, or 3. expression of sequestered or cryptic antigen through membrane changes or rearrangement. The rapid rate of appearance of Thy-1.2 (fifteen minutes) suggests that the appearance may be due to modification of existing compounds or expression of a cryptic antigen rather than *de novo* synthesis. This hypothesis has been tested using neuraminidase in conjunction with anti-Thy-1.2 and anti-$G_{M1}$ antisera.

Neuraminidase has been reported to expose antigens specified by autologous antibodies, histocompatability antigens on lymphocytes, tumor antigens and a number of other antigens on a variety of cell types. Neuraminidase has also been reported to expose cholera toxin receptor ($G_{M1}$ ganglioside) on liver cells (122) and adrenal cells (157). The reported experiments have shown that *Vibrio cholerae* neuraminidase can expose or cause the expression of Thy-1,2 and $G_{M1}$ antigens on bone marrow stem cells which have been separated on BSA gradients. The expression of $G_{M1}$ and Thy-1,2 with neuraminidase treatment occurs at a rate similar to expression induced by thymic factor (Fig. 1.). Minor differences were observed in the relative rate of appearance of Thy-1,2 with neuraminidase or thymic factor treatment after fifteen minutes of incubation but the maximum amount of cytotoxicity observed at sixty minutes was similar regardless of the means of expression.

Interpretation of data involving neuraminidase treatment of cells is complicated by at least two factors. First, neuraminidase may bind to cells in a specific way giving rise to a "new" antigen on the cells which may be recognized by preformed anti-neuraminidase antibodies in sera. Second, neuraminidase treatment may expose antigens which are common to all cells and which are recognized by autologous antibodies. Sedlacek and Seiler (158) reported that VCN binds irreversibly to human peripheral lymphocytes after brief VCN treatment. VCN remains bound after four washings and was observed using anti-VCN antisera and FITC-conjugated goat anti-rabbit Ig. It appears likely that the binding involves the active site because heat-denatured VCN does not

bind to cells. This type of binding could account for an "autologous" antibody binding to VCN treated cells observed in many human and animal antisera. In the present study the possibility that VCN binding to cells was responsible for increased cytotoxicity was eliminated because cytotoxicity was observed only in the 21 and 23% BSA and, more importantly, VCN treated AKR (Thy-1.1) marrow cells were not killed by anti-Thy-1.2 antisera.

The possibility that VCN treatment exposes determinants that are recognized by autologous sera has been raised by several investigators. It has been reported that VCN reveals determinants which are recognized by autologous, undiluted normal human sera (77,143). The same determinants appeared on all human cell types which they tested, including erythrocytes, B and T lymphocytes, monocytes, leukemic lymphocytes and cells of lymphoid lines. Similar autologous antibodies have been observed for various mouse strains (159). These normal mouse sera, in concentrated form, exhibited cytotoxicity for every murine cell line tested. It is proposed that the Thy-1.2 and $G_{M1}$ antigenicity of VCN treated bone marrow cells was not due to a non-specific determinant, for several reasons: 1. Antisera used in the experiments were generally more diluted (at least 1/20 or 1/40) than autologous antisera usually used to detect nonspecific VCN exposed antigen: 2. Normal mouse sera, (collected from bone marrow donor mice) which was used for controls, did not have non-specific cytotoxicity for either marrow cells or VCN treated cells, and the cytotoxicity of anti-H-2 antisera did not increase after VCN treatment; 3. The cytotoxicity of anti-Thy-1.2 or anti-$G_{M1}$ was specific exclusively for

VCN or thymic factor-treated cells of the 19-21% and 21-23% BSA layer and not for VCN or thymic factor-treated cells of other BSA layers; and 4. Anti-Thy-1.2 antisera retained allogeneic specificity for VCN treated cells of the Thy-1.2 allotype.

The possibility has been raised (81) that VCN treatment of cells might cause a greater susceptibility of the cells to complement action. Increase in cytotoxicity of antisera for VCN-treated cells has been observed for some antigens which were already present on the cell surface. The reported experiments show the appearance of cytotoxicity of antisera for VCN marrow cells which did not previously express antigenic specificity. Comparable cytotoxicity results were obtained from either VCN treatment or treatment with thymic factor. Furthermore, it was concluded that the VCN treated marrow cells were not more susceptible to complement lysis, because under the conditions of the assay, lysis by anti-H-2 antisera was not increased.

It has been proposed that nude mouse splenocytes contain some Thy-1.2 bearing cells even though these cells are not susceptible to lysis by anti-Thy-1.2 antisera (159). The results support the presence of Thy-1.2 antigen (as well as $G_{M1}$) on these cells because the antigens were expressed after brief neuraminidase treatment or after incubation with thymic factor (Table V). It is possible that Thy-1.2 antigen is sequestered on nude mouse splenocytes and C3H marrow stem cells in the same way and the expression of Thy-1.2 on marrow stem cells and on nude mouse splenocytes proceeds in a similar manner.

Schlesinger (24) has proposed that the quantity of Thy-1.2 could not be increased on murine splenocytes, thymocytes or lymph node cells by neuraminidase treatment. If the Thy-1.2 antigen on these cells is maximally expressed, neuraminidase treatment would have no effect. Alternatively, using mixed populations of cells may obscure an increase in a small population of cells. In this report, a fraction of bone marrow cells was used which was enriched in stem cells and which does not normally express T cell differentiation antigens. Thus it was possible to observe increases in Thy-1 positive cells which make up a small fraction of the total population of bone marrow cells.

Based on evidence that cholera toxin binds to $G_{M1}$ ganglioside (148,149) and that it binds specifically to murine lymphocytes (161, 162), it was possible to predict that pretreating anti-$G_{M1}$ susceptible lymphocytes with cholera toxin would abrogate the cytotoxicity of anti-$G_{M1}$ antisera. This proposal has been confirmed by incubating thymic factor treated marrow stem cells with cholera toxin before addition of anti-$G_{M1}$ serum (Table V). The cytotoxicity of anti-Thy-1.2 was also blocked by pretreatment of Thy-1.2 positive cells with cholera toxin or choleragenoid. The blocking effect of cholera toxin was also confirmed in experiments with thymocytes (Table VI). The finding that cholera toxin and choleragenoid, which bind specifically to $G_{M1}$ ganglioside, can block the cytotoxicity of anti-$G_{M1}$ serum as well as anti-Thy-1.2 serum suggests that $G_{M1}$ and Thy-1.2 are immunologically related and supports previous findings. It should be noted, however, that the possibility that the blocking of anti-Thy-1.2 sera occurs through steric hindrance of Thy-1.2 antigen cannot be eliminated with these experiments.

The properties of anti-$G_{M1}$ antisera with respect to murine lymphocytes parallel anti-Thy-1.2 activity in several ways. 1. Anti-$G_{M1}$ and anti-Thy-1.2 antisera were only slightly cytotoxic for bone marrow cells but became significantly cytotoxic for these cells after the cells were incubated for brief periods with thymic factor or neuraminidase. 2. These antisera were primarily cytotoxic for neuraminidase or thymic factor-treated bone marrow cells derived from the 19-21% and 21-23% BSA layer of a discontinuous gradient. 3. Anti-$G_{M1}$ and anti-Thy-1.2 were not cytotoxic for nude mouse splenocytes, unless these cells were pretreated with neuraminidase or thymic factor. 4. The cytotoxicity of anti-$G_{M1}$ or anti-Thy-1.2 could be blocked by pretreatment of thymic factor-treated marrow cells (or thymocytes) with cholera toxin which binds $G_{M1}$ ganglioside.

It should be noted that $G_{M1}$ ganglioside from lymphoid sources has not been fully identified although it co-chromatographs with brain $G_{M1}$. The water soluble glycolipids (containing $G_{M1}$) of lymphocytes are a complex mixture of gangliosides and large oligoglycolipids and often contain glucosamine and fucose rather than galactosamine found in brain gangliosides. Barton and Rosenberg (74), have shown that *Vibrio cholerae* neuraminidase does not hydrolyze the sialic acid of pure $G_{M1}$ ganglioside and that the gangliosides of the intact cell surfaces are deeply localized and sterically inaccessible to VCN. They proposed that sialic acid released from intact cells is from surface sialoglycoproteins rather than gangliosides.

The chemical nature of Thy-1.2 is not completely resolved. Evidence presented here of blocking by cholera toxin supports the

glycolipid nature of the antigen, but reports have appeared presenting evidence for protein nature of the antigen (10,23). It is possible that Thy-1.2 antigenicity resides in oligosaccharides which may be attached to membrane sphingolipid and protein. The cytotoxicity of anti-Thy-1.2 and anti-$G_{M1}$ antisera after neuraminidase treatment may be due to either glycolipid or glycoprotein antigens similar in nature to Thy-1.2.

The similarity in the rate of appearance of anti-Thy-1.2 and anti-$G_{M1}$ reactive determinants on either VCN or thymic factor-treated marrow cells suggests that the thymic factor-induced appearance of Thy-1.2 and $G_{M1}$ may proceed in a similar manner. Neuraminidase activity has not, however, been detected in the thymic factor preparation used in this study. It is possible that there is endogenous cell surface neuraminidase which is activated by thymic factor. Based on the evidence presented herein, it is proposed that Thy-1.2 and $G_{M1}$ antigen are present on marrow stem cells in a cryptic state where they may be rapidly expressed through membrane rearrangement or by alteration of a Thy-1.2 precursor (e.g. $G_{D1a}$).

# BIBLIOGRAPHY

BIBLIOGRAPHY


1. Reif, A.E. and Allen, J.M.V. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissues. J. Exp. Med. 120:413-433.

2. Douglas, T.C. 1972. Occurence of a theta-like antigen in rats. J. Exp. Med. 136:1054-1062.

3. Stern, P. L. 1973. Theta alloantigen on mouse and rat fibroblasts. Nature (New Biol.) 247:76-78.

4. Scheid, M., Boyse, E.A., Carswell, E.A., Old, L.J. 1972. Seriologically demonstrable alloantigens of mouse epidermal cells. J. Exp. Med. 135:938-955.

5. Reig, A.E. and Allen, J.M. 1966. Mouse thymic iso-antigens. Nature 209:521.

6. Raff, M.D. 1969. Theta isoantigen as a marker of thymus derived lymphocytes in mice. Nature 224:378-379.

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

8. Schwartz, B.D. and Nathenson, S.G. 1971. Isolation of H-2 alloantigens solubilized by the detergent NP-40. J. Immunol. 107:1363-1367.

9. Vitetta, E.S., Bianco, C. Nussenzwig, B. and Uhr, J.W. 1972. Cell surface immunoglobulin IV. Distribution among thymocytes, bone marrow cells, and their derived populations. J. Exp. Med. 136:181-193.

10. Kuchich, U.N., Bennett, J.C., and Johnson, B.J. 1975. The protein nature of the Thy-1.2 alloantigen as expressed by the murine lymphoblastoid line S-49.1TB.2.3. J. Immunol. 115;626-630.

11. Letarte-Muirhead, M., Acton, R.T. and Williams, A.F. 1974. Preliminary characterization of Thy-1.1 and Ag-B antigens from rat tissues solubilized in detergents. Biochem. J. 143:51-61.

12. Barclay, A.N., Letarte-Muirhead, M., and Williams, A.F. 1975. Purification of the Thy-1 molecule from rat brain. Biochem. J. 151:699-706.

13. Barclay, A.N., Letarte-Muirhead, M., Williams, A.F. and Faulkes, R.A. 1976. Chemical characterization of the Thy-1 glycoproteins from the membranes of rat thymocytes and brain. Nature 263:563-567.

14. Morris, R.J., Letarte-Muirhead, M. and Williams, A.F. 1975. Analysis in deoxycholate of three antigenic specificities associated with rat Thy-1 molecule. Eur. J. Immunol. 5:282-285.

15. Esselman, W.J. and Miller, H.C. 1974. Brain and thymus lipid inhibition of antibrain-associated theta-cytotoxicity. J. Exp. Med. 139:445-450.

16. Miller, H.C. and Esselman, W.J. 1975. Modulation of the immune response by antigen reactive lymphocytes following cultivation with gangliosides. J. Immunol. 115:839-843.

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

18. Vitetta, E.S., Boyse, E.A. and Uhr, J.W. 1973. Isolation and characterization of a molecular complex containing Thy-1 antigen from the surface of murine thymocytes and T cells. Eur. J. Immunol. 3:446-453.

19. Vitetta, E.S., Uhr, J.W. and Boyse, E.A. 1974. Metabolism of H-2 and Thy-1 alloantigens in murine thymocytes. Eur. J. Immunol. 4:276-282.

20. Hartmann, K.U. 1970. Induction of a hemolysin response in vitro; interaction of cells of bone marrow origin and thymic origin. J. Exp. Med. 132:1267-1278.

21. Bloom, B.R. 1971. In vitro approaches to the mechanism of cell-mediated immune reactions. Adv. Immunol. 13:102.

22. Atwell, J.L., Cone, R.E. and Marchalonis, J.J. 1973. Isolation of theta antigen from the surface of thymus lymphocytes. Nature (New Biol.) 241:251-252.

23. Johnson, B.J., Kuchich, U.N. and Maurelli, A.T. 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. 116:1669-1672.

24. Schlesinger, M. and Gottesfeld, S. 1971. The effect of neuraminidase on the expression of cellular antigens. Trans. Proc. 3:1151.

25. Trainin, N. and Small, M. 1970. Studies on some physiochemical properties of a thymus humoral factor conferring immunocompetence on lymphoid cells. J. Exp. Med. 132:885-891.

26. Small, M. and Trainin, N. 1967. Increase in antibody-forming cells of neonatally thymectomized mice receiving thymus extract. Nature 216:377-379.

27. Trainin, N. and Linker-Israeli, M. 1967. Restoration of immunologic reactivity of thymectomized mice by calf thymus extracts. Cancer Res. 27:309-313.

28. Bach, J.F., Papiernik, M., Levasseur, P., Dardenne, M., Barvis, A. and LeBrigand, H. 1972. Evidence for a serum factor secreted by the human thymus. Lancet 2:1056-1058.

29. Komuro, K. and Boyse, E.A. 1973. In-vitro demonstration of thymic hormone in the mouse by conversion of precursor cells into lymphocytes. Lancet 1:740-743.

30. Boyse, E.A. and Old, L.J. 1969. Some aspects of normal and abnormal cell surface genetics. Ann. Rev. Genet. 3:269.

31. Itakura, K., Hutton, J.J., Boyse, E.A. and Old, L.J. 1972. Genetic linkage relationships of loci specifying differentiation alloantigens in the mouse. Transplantation 13:239-243.

32. Shigeno, N., Hammerling, U., Arpels, C., Boyse, E.A. and Old, L.J. 1968. Preparation of lymphocyte-specific antibody from anti-lymphocyte serum. Lancet 2:320.

33. Stockert, E., Old, L.J. and Boyse, E.A. 1971. The $G_{IX}$ system: A cell surface alloantigen associated with murine leukemia virus; implications regarding chromosomal integration of the viral genome. J. Exp. Med. 133:1334.

34. Scheid, M.P., Hoffmann, M.K., Komuro, K., Hammerling, U., Abbott, J., Boyse, E.A., Cohen, G.H., Hooper, J.A., Schulof, R.S. and Goldstein, A.L. 1973. Differentiation of T cells induced by preparations from thymus and by nonthymic agents. J. Exp. Med. 138:1027-1032.

35. Goldstein, A., Guha, A., Zatz, M., Hardy, M. and White, A. 1972. Purification and biological activity of thymosin, a hormone of the thymus gland. Proc. Natl. Acad. Sci. (USA) 69:1800-1803.

36. Kook, A.I., and Trainin, N. 1974. Hormone-like activity of a thymus humoral factor on the induction of immune competence in lymphoid cells. J. Exp. Med. 139:193-207.

37. Bach, M.A., Bach, and Bach J.F. 1973. Studies on thymus products VI. The effect of cyclic necleotides and prostaglandins on rosette-forming cells; interactions with thymic factor. Eur. J. Immunol. 3:778-783.

38. Scheid, M.P., Goldstein, G., Hammerling, U. and Boyse, E.A. 1975. Lymphocyte differentiation from precursor cells in vitro. Ann. N.Y. Acad. Sci. 249:530-540.

39. Bach, M.A., Fournier, C. and Bach, J.F. 1975. Regulation of theta antigen expression by agents altering cyclic AMP levels and by thymic factor. Ann. N.Y. Acad. Sci. 249:316-327.

40. Goldstein, G. 1974. Isolation of bovine thymin: a polypeptide hormone of the thymus. Nature 247:11-14.

41. Goldstein, G., Scheid, M., Hammerling, U., Boyse, E.A., Schlesinger, D.H. and Niall, H.D. 1975. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc. Natl. Acad. Sci. (USA) 72:11-15.

42. Storrie, B., Goldstein, G., Boyse, E.A. and Hammerling, U. 1976. Differentiation of thymocytes: evidence that induction of the surface phenotype requires transcription and translation. J. Immunol. 116:1358-1362.

43. Rotter, V. and Trainin, N. 1975. Increased mitogenic reactivity of normal spleen cells to T lectins induced by thymic humoral factor (THF). Cell. Immunol. 16:413-421.

44. Basch, R. and Goldstein, G. 1975. Antigenic and functional evidence for the in vitro inductive activity of thymopoietin (thymin) on thymocyte precursors. Ann. N.Y. Acad. Sci. 249:290-299.

45. Basch, R.S. and Goldstein, G. 1975. Thymopoietin-induced acquisition of responsiveness to T cell mitogens. Cell. Immunol. 20:218-228.

46. Schlesinger, D.H., Goldstein, G., Scheid, M.P. and Boyse, E.D. 1975. Chemical synthesis of a peptide fragment of TPII that induces selective T cell differentiation. Cell 5:367-370.

47. Schlesinger, D.H. and Goldstein, G. 1975. The amino acid sequence of thymopoietin II. Cell 5:361-365.

48. Till, J.E., McCulloch, E.A. and Siminovitch, L. 1964. A Stochastic model of stem cell proliferation based on growth of spleen colony-forming cells. Proc. Natl. Acad. Sci. (USA) 51:29.

49. Osmond, D.G. and Nossal, G.J.V. 1974. Differentiation of lymphocytes in mouse bone marrow. Cell. Immunol. 13:117.

50. Yoshida, Y., and Osmond, D.G. 1971. Graft vs. host activity of rat bone marrow, marrow fractions and lymphoid tissue. Transplantation 12:121.

51. Osmond, D.G. and Yoshida, Y. 1971. Blastogenic transformation in lymphocyte-rich fractions of guinea pig and rat bone marrow. In Proc. of the Fourth Annual Leukocyte Culture Conference. (Ed: R. McIntyre) page 97. Appleton Century Crafts, N.Y.

52. El-Arini, M.O. and Osoba, D. 1973. Differentiation of Thymus-derived cells from precursors in bone marrow. J. Exp. Med. 137:825.

53. Loor, F. and Roelants, G. 1975. Immunofluorescence studies of a possible prethymic T-cell differentiation in congenitally athymic (nude) mice. Ann. N.Y. Acad. Sci. 254:226-242.

54. Turner, R.W.A., Siminovitch, L., McCulloch, E.A. and Till, J.E. 1967. Density gradient centrifugation of hemopoietic colony-forming cells. J. Cell Physiol. 69:73-82.

55. Boyse E.A. and Bennett, D. 1974. In Cellular Selection and Regulation in the Immune Response. (Ed: G.M. Edelman) Raven Press, N.Y. page 164.

56. Bach, J.F. and Carnaud, C. 1976. Thymic factors. Progr. Allergy 21:342-408.

57. Basch, R. and Kadish, J. 1977. Hematopoietic thymocyte precursors II. Properties of precursors. J. Exp. Med. 145:405-419.

58. Rambourg, A. 1971. Morphologic and histochemical aspects of glycoproteins at the surface of animal cells. Int. Rev. Cytol. 31:57-114.

59. Parsons, D.F. and Subjeck, J.R. 1972. The morphology of the polysaccharide coat of mammalian cells. BBA 265:85-113.

60. Drzeniek, R. 1967. Differences in splitting capacity of virus and V. cholerae neuraminidases on sialic acid type substrates. Biochem. Biophys. Res. Comm. 26:631-638.

61. Ambrose, E.J. 1966. Alteration of cell charge after neuraminidase treatement. Progres. Biophys. 16:241.

62. Lee, A. 1968. Effect of neuraminidase on the phagocytosis of heterologous red cells by mouse peritoneal macrophages. Proc. Soc. Exp. Biol. Med. 128:891-894.

63. Weiss, L. Mayhew, E. and Ulrich, K. 1966. The effect of neuraminidase on the phagocytic process in human monocytes. Lab Invest. 15:304-309

64. Baylor, M.E. 1964. Neuraminidase alteration of viral hemagglutination. Trans. N.Y. Acad. Sci. 26:1103.

65. Woodruff, J.J. and Gesner, B.M. 1969. The effect of neuraminidase on the fate of transfused lymphocytes. J. Exp. Med. 129:551-567.

66. Yamakawa, T. and Suzuki, S. 1951. The chemistry of the lipids of post-hemolytic residue or stroma of erythrocytes. I. Concerning the ether insoluble lipids of lyophilized horse blood stroma. J. Biochem (Tokyo) 38:199-212.

67. Yamakawa T. and Suzuki, S. 1953. The chemistry of the lipids of post-hemolytic residue or stroma of erythrocytes. IV. Distribution of lipid-hexosamine and lipid-hemataminic animals. J. Biochem. (Tokyo) 40:7-10.

68. Currie, G.H. and Bagshawe, K.D. 1968. The role of sialic acid in antigenic expression. Brit. J. Cancer 22;843.

69. Aminoff, D., Bell, W.C., Fulton I. and Ingebrigtsen, N. 1976. Effect of sialidase on the viability of erythrocytes in circulation. Am. J. Hematol. 1:419-432.

70. Barth, R.F., and Singla, O. 1974. Alterations in the immunogenicity and antigenicity of mammalian erythrocytes following treatment with neuraminidase. Proc. Soc. Exp. Biol. Med. 145:168.

71. Gregoriadis, G., Putnam, D., Louis, L. and Neerunjun, D. 1974. Comparative effect and fate of non-entrapped and liposome-entrapped neuraminidase injected into rats. Biochem. J. 140:323-330.

72. Schlesinger, M. and Galili, U. 1974. The effect of neuraminidase on the formation of E-rosettes by human peripheral lymphocytes and sheep red blood cells. Behring Inst. Mitt. 55:272.

73. Schlesinger, M. and Amos, B.D. 1971. Effect of neuraminidase on serologic properties of murine lymphoid cells. Transp. Proc. 3:895.

74. Barton, N.W. and Rosenberg, A. 1973. Action of Vibrio cholerae neuraminidase (sialidase) upon the surface of intact cells and their isolated sialolipid components. J. Biol. Chem. 248:7353-7358.

75. Reisner, E.G., Flye, M.W., Chung, K.S.S. and Amos, D.B. 1974. The cytotoxic reactivity and sialic acid content of human lymphoid cells. Tissue Antigens 4:7-20.

76. Rosenberg, S. and Rogentine, G.N. 1972. Natural human antibodies to "hidden membrane components." Nature 239:203.

77. Rogentine, G.N. and Plocinik, B.A. 1974. Carbohydrate inhibition studies of the naturally occurring antibody to neuraminidase-treated human lymphocytes. J. Immunol. 113:848.

78. Grothaus, E.A., Flyde, M. W., Yunis, E.J. and Amos, D.B. 1971. Human lymphocyte antigen reactivity modified by neuraminidase. Science 173:542.

79. Schlesinger, M. and Gottesfeld, S. 1971. The effect of neuraminidase on expression of cellular antigens. Transp. Proc. 3:1151-1154.

80. Rosenburg, S.A., Plocinik, B.A. and Rogentine, G.N. 1972. Unmasking of human lymphoid cell heteroantigens by neuraminidase treatment. J. Natl. Cancer Inst. 48:1271-1276.

81. Reisner, E.G. and Amos, D.B. 1972. The complement-binding and absorptive capacity of human white blood cells treated with neuraminidase. Transplantation 14:455-461.

82. Burnet, F.M. and Anderson, S.G. 1947. The "T" antigen of guinea pig and human red blood cells. Aust. J. Exp. Biol. Med. Sci. 25:213.

83. Mollison, P.L. 1970. The role of complement in antibody-mediated red-cell destruction. Brit. J. Hematol. 18:249-255.

84. Ray, P.K., Gewurz, H. and Simmons, R.L. 1970. The mechanism of increased sensitivity of neuraminidase-treated cells to antibody induced cytolysis. Fed. Proc. (abstr.) 29:573.

85. Ray, P.K. and Simmons, R.L. 1971. Failure of neuraminidase to unmask allogeneic antigens on cell surfaces. Proc. Soc. Exp. Med. 138:600-604.

86. Gray, B.N. 1976. Naturally occurring human antibody to neuraminidase-treated human lymphocytes. J. Natl. Cancer Inst. 56:211.

87. Seiler, F.R. and Sedlacek, H.H. 1974. Alterations of Immunological phenomena by neuraminidase; marked rise in the number of lymphocytes forming rosettes or bearing immunoglobulin receptors. Behring Inst. Mitt. 55:258-271.

88. Kirby, D.R.S., Billington, W.D., Bradbury, S. and Goldstein, D.J. 1964. Antigen barrier of the mouse placenta. Nature (London) 204:548-549.

89. Bradbury, S., Billington, W.D. and Kirby, D.R.S. 1965. A histochemical and electron microscopal study of the fibrinoid of the mouse placenta. J.R. Microsc. Soc. (J. Microscopy, Oxford) 84:199-211.

90. Warren, G.H., Williams, E.C., Alburn, H.E. and Seifter, J. 1949. Rous chicken sarcoma as a source for hyaluronic acid. Archs. Biochem. 20:300-304.

91. Girshman, E. 1952. Histochemical analysis of mucopolysaccharides occurring in mucus-producing tumors. Cancer 5:700-707.

92. Franks, L.M., Oshea, J.D. and Thomson, A.E.R. 1964. Mucin in the prostate: A histochemical study in normal glands, latent, clinical and colloid cancer. Cancer 17:983-991.

93. Defendi, V. and Gasic, G. 1963. Surface mucopolysaccharides of polyoma virus transformed cells. J. Cell. Physiol. 62:23-26.

94. Hughes, R.C., Palmer, P.D. and Sanford, B.H. 1973. Factors involved in the cytotoxicity of normal guinea pig serum for cells of murine tumor TA3 sublines treated with neuraminidase. J. Immunol. 111:1071-1080.

95. Lundgren, G., Jeitz, L., Lundin, L. and Simmons, R.L. 1971. Increased stimulation by neuraminidase-treated cells in mixed lymphocyte cultures. Fed. Proc. (abstr.) 30:395.

96. Flye, M.W., Reisner, E.G. and Amos, D.B. 1973. The in vitro effect of neuraminidase on human lymphocytes. J. Surg. Res. 15:96.

97. Sanford, B. 1967. An alteration in tumor histocompatability induced by neuraminidase. Transplantation 5:1273-1279.

98. Currie, G.A. and Bagshawe, K.D. 1969. Tumor specific immunogenicity of methylcholanthrene-induced sarcoma cells after incubation with neuraminidase. Brit. J. Cancer 23:141-149.

99. Simmons, R.L., Rios, A. and Ray, P.K. 1970. Mechanism of neuraminidase induced antigen "unmasking." Surg. Forum 21:265-267.

100. Simmons, R.L. 1971. Immunotherapy of cancer; immunospecific rejection of tumors in recipients of neuraminidase-treated tumor cells plus BCG. Science 258:62.

101. Sanford, B.H. 1974. Neuraminidase and cancer therapy: possible mechanisms of action. Behring. Inst. Mitt. 55:306-308.

102. Carubelli, R., Trucco, R.E. and Caputto, R. 1976. Neuraminidase activity in mammalian organs. Biochim. Biophys. Acta 60:196-197.

103. Horvat, A. and Touster, O. 1968. On the lysosomal occurrence and the properties of the neuraminidase of rat liver and of Erlich ascites tumor cells. J. Biol. Chem. 243:4380-4390.

104. Yeh, A.K., Tulsiana, D.R.P. and Carubelli, R. 1971. Neuraminidase activity in human leukocytes. J. Lab. Clin. Med. 78:771-778.

105. Taha, B.H. and Carubelli, R. 1967. Mammalian neuraminidase: intracellular distribution and changes of enzyme activity during lactation. Arch. Biochem. Biophys. 119:55-61.

106. Tulsiani, D.R.P. and Carubelli, R. 1970. Studies on the soluble and lysosomal neuraminidases of rat liver. J. Biol. Chem. 245: 1821-1827.

107. W. Gielin. 1974. Neuraminidase in higher organisms. Behring Inst. Mitt. 55:85-88.

108. Schengrund, C.L., Jensen, D.S. and Rosenburg, A. 1972. Localization of sialidase in the plasma membrane of rat liver cells. J. Biol. Chem. 247:2742-2746.

109. Tettamanti, G., Preti, A., Lombardo, A., Suman, T. and Zambotti, V. 1975. Membrane-bound neuraminidase in the brain of different animals: Behavior of the enzymes on endogenous sialo derivatives and rationale for its assay. J. Neurochem. 25:451-456.

110. Yohe, H.C. and Rosenburg, A. 1977. Action of intrinsic sialidase of rat brain synaptic membranes on membrane sialolipid and sialoprotein components in situ. J. Biol. Chem. 252:2412-2418.

111. Finkelstein, R.A. and LoSpalluto, J.J. 1969. Pathogenesis of experimental cholera. Preparation and isolation of choleragen and choleragenoid. J. Exp. Med. 130:185-202.

112. LoSpalluto, J.J. and Finkelstein, R.A. 1972. Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid) Biochim. Biophys. Acta 257:158-166.

113. Finkelstein, R.A., Noms, H.T. and Dutta., N.K. 1964. Pathogenesis of experimental cholera in infant rabbits. J. Inf. Dis. 114:203.

114. vanHeyningen, W.E., Carpenter, C.C.J., Pierce, N.F. and Greenough, W.B. III. 1971. Deactivation of cholera toxin by ganglioside. J. Inf. Dis. 124:415:418.

115. Baker, A.L., Kaplan, M.M., Kimberg, D.V. and Pierce, N.F. 1971. Stimulation of rat liver enzyme activity by intravenous cholera toxin. Gastroenterology 60:739.

116. Franks, D.J. 1976. Cholera toxin and adenylate cyclase; properties of activated enzyme in liver plasma membranes. Can. J. Biochem. 54:981-987.

117. Donta, S.T., King, M. and Sloper, K. 1973. Induction of steroidogenesis in tissue culture by cholera enterotoxin. Nature (New Biol)243:246-247.

118. Hollenberg, M.D. and Cuatrecasas, P. 1973. Epidermal growth factor: receptors in human fibroblasts and modulation of action by cholera toxin. Proc. Natl. Acad. Sci. (USA) 70:2964-2968.

119. Cuatrecasas, P. 1973. Cholera toxin-fat cell interaction and the mechanism of activation of the lipolytic response. Biochem. 12:3567-77.

120. Vaughn, M., Pierce, N. and Greenough, W.B. 1970. Stimulation of glycerol production in fat cells by cholera toxin. Nature 226:658-659.

121. Moritz, M., Iber, F.L. and Moore, E.W. 1974. Rabbit cholera: effects of cycloheximide on net water and ion fluxes and transmural electrical potentials. Gastroenterology 63:76-82.

122. Cuatrecasas, P. 1973. Interaction of Vibrio cholerae enterotoxin with cell membranes. Biochem. 12:3547-3558.

123. Pierce, N.F. 1973. Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxins of Vibrio cholerae and Escherichia coli. J. Exp. Med. 137:1009-1023.

124. Holmgren, J., Lonnroth, I. and Svennerholm, L. 1973. Fixation and inactivation of cholera toxin by $G_{M1}$ ganglioside. Scand. J. Inf. Dis. 5:77-78.

125. Hollenberg, M.D., Fishman, P.H., Bennett, V. and Cuatrecasas, P. 1974. Cholera toxin and cell growth: role of membrane gangliosides. Proc. Natl. Acad. Sci. 71:4224-4228.

126. Cuatrecasas, P. 1973. Vibrio cholera choleragenoid: Mechanism of inhibition of cholera toxin action. Biochem. 12:3577-3581.

127. Lonnroth, I. and Holmgren, J.J. 1973. Subunit structure of cholera toxin. J. Gen. Microb. 76:417-427.

128. Bennett, V., O'Keefe, E. and Cuatrecasas, P. 1975. Mechanism of action of cholera toxin and the mobile receptor theory of hormone-receptor adenylate cyclase activity. Proc. Natl. Acad. Sci. (USA) 72:33-37.

129. Bennett, V. and Cuatrecasas, P. 1975. Mechanism of activation of adenylate cyclase by V. Cholerae enterotoxin. J. Membr. Biol. 22:29-52.

130. Craig, S.W. and Cuatrecasas, P. 1975. Mobility of cholera toxin receptors on rat lymphocyte membranes. Proc. Natl. Acad. Sci. (USA) 72:3844-3848.

131. Cuatrecasas, P. 1973. Isolation and characterization of hormone receptors. Neurosci. Res. Program. Bull. 11:215-219.

132. Guerrant, R.L., Chen, L.C. and Sharp, G.W.G. 1972. Intestinal adenyl-cyclase activity in canine cholera: correlation with fluid accumulation. J. Inf. Dis. 125:377-381.

133. Singer, S.J. and Nicolson, G.L. 1972. The fluid mosaic model of the structure of cell membranes. Science 175:720-731.

134. vanHeyningen, S. 1974. Cholera toxin: interaction of subunits with ganglioside $G_{M1}$. Science 183:656-657.

135. Gill, D.M. and King, C.A. 1975. The mechanism of action of cholera toxin in pigeon erythrocytes lysates. J. Biol. Chem. 250:6424-6432.

136. Kandel, J., Collier, R.J. and Chung, D.W. 1974. Interaction of fragment A from diptheria toxin with nicotinamide adenine dinucleotide. J. Biol. Chem. 249:2088-2097.

137. Moss, J. 1977. Mechanism of action of choleragen: Evidence for ADP-ribosyltransferase activity with arginie as an acceptor. J. Biol. Chem. 7:2455(comm.).

138. Flores, J., Witkum, P. and Sharp, G.W.G. 1976. Activation of adenylate cyclase by cholera toxin in rat liver homogenates. J. Clin. Invest. 57:450-458.

139. Sultzer, B.M. and Craig, J.P. 1973. Cholera toxin inhibits macromolecular synthesis in mouse spleen cells. Nature (New Biol.) 244:179-180.

140. Honjo, T., Nishizuka, Y., Kato, I. and Hayaishi, O. 1971. Adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by diptheria toxin. J. Biol. Chem. 246:4251-4260.

141. Moss, J., Richards, R.L., Alving, C. and Fishman, P.R. 1977. Effect of the A and B protomers of choleragen on release of trapped glucose from liposomes containing or lacking ganglioside $G_{M1}$. J. Biol. Chem. 252:797-798 (comm.).

142. Stein-Douglas, K.E., Schwarting, G.A., Naiki, M. and Marcus, D.M. 1976. Ganglioside as a marker for murine lymphocyte subpopulations. J. Exp. Med. 143:822-832.

143. Winchester, R.J., ShuManFu, Winfield, J.B. and Kunkel, H.G. 1975. Immunofluroescent studies on antibodies directed to a buried membrane structure present in lymphocytes and erythrocytes. J. Immunol. 114:410-414.

144. Ray, P.K. and Simmons, R.L. 1973. Serological studies of enzyme-treated murine lymphoid cells. Proc. Soc. Exp. Biol. Med. 142: 846-852.

145. Rosenberg, S.A., Schwartz, S. and Baker, A. 1974. Natural antibodies to "cryptic" membrane antigens exposed by treatment with neuraminidase. Behring Inst. Mitt. 55:204-208.

146. Hakormori, S. 1969. Differential reactivities of fetal and adult human erythrocytes to antisera directed against glycolipids of human erythrocytes. Vox. Sang. 16:478-485.

147. Gahmberg, C.T. and Hakomori, S. 1973. External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. J. Biol. Chem. 248:4311-4317.

148. Homlgren, J., Lonnroth, I. and Svennerholm, L. 1973. Tissue receptor for cholera exotoxin: postulated structure from studies with $G_{M1}$ ganglioside and related glycolipids. Infect. Immun. 8:208-214.

149. King, C.A. and vanHeyningen, W.E. 1973. Deactivation of cholera toxin by a sialidase-resistant monosialosyl-ganglioside. J. Inf. Dis. 127:639-647.

150. Finkelstein, R.A., Boesman, M., SimHeeNeoh, LaRue, M.K. and Delaney, R. 1974. Dissociation and recombination of the subunits of the cholera enterotoxin (choleragen). J. Immunol. 113:145-150.

151. Miller, H.C. and Esselman, W.J. 1975. Identification of theta-bearing T-cells derived from bone marrow cells treated with thymic factor. Ann. N.Y. Acad. Sci. 245:54-60.

152. Esselman, W.J. and Miller, H.C. 1974. The ganglioside nature of theta antigens. Fed. Proc. (abstr.) 33:771.

153. Dicke, K.A., Tridente, G. and vanBekkum, D.W. 1969. The selective elimination of immunologically competent cells from bone marrow and lymphocyte cell mixtures. Transplantation 8:422-434.

154. Naiki, M., Marcus, D. and Ledeen, R. 1974. Properties of antisera to ganglioside $G_{M1}$ and asialo $G_{M1}$. J. Immunol. 113:84-93.

155. Finkelstein, R.A. and LoSpalluto, J.J. 1970. Production of highly purified choleragen and choleragenoid. J. Inf. Dis. 121 (Supplement):63-72.

156. Komuro, K. and Boyse, E.A. 1973. Induction of T lymphocytes from precursor cells in vitro by a product of the thymus. J. Exp. Med. 138:479-482.

157. Haksar, A., Maudsley, D.V. and Peron, F.G. 1974. Neuraminidase treatment of adrenal cells increases their response to cholera enterotoxin. Nature 251:514-515.

158. Sedlacek, H.H. and Seiler, F.R. 1974. Demonstration of Vibrio cholerae neuraminidase (VCN) on the surface of VCN-treated cells Behring Inst. Mitt. 55:254-257.

159. Ray, P.K. and Simmons, R.L. 1972. Comparative effect of viral and bacterial neuraminidase on the complement sensitivity of lymphoid cells. Clin. Exp. Immunol. 10:139-150.

160. Roelants, G.E., Loor, F., vonBoehmer, H., Sprent, J., Hagg, L., Mayor, K.S. and Ryden, A. 1975. Five types of lymphocytes characterized by double immunofluroescence and electrophoretic mobility. Organ distribution in normal and nude mice. Eur. J. Immunol. 5:127-131.

161. Holmgren, J. 1973. Comparison of the tissue receptors for Vibrio cholerae and Escherichia coli enterotoxins by means of gangliosides and natural cholera toxoid. Infect. Immun. 8:851-859.

162. Trowbridge, I.S., Weissman, I.L. and Bevan, M.J. 1975. Mouse T-cell surface glycoprotein recognized by heterologous antithymocyte sera and its relationship to Thy-1 antigen. Nature 256:652-654.