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OF THE THY-1 DIFFERENTIATION
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STUDIES ON THE IN VITRO INDUCTION OF THE THY-1 DIFFERENTIATION ANTIGEN ON MURINE PROTHYMOCYTES

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ABSTRACT

STUDIES ON THE IN VITRO INDUCTION

OF THE THY-1 DIFFERENTIATION

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The induction of Thy-1, a T cell differentiation antigen, on murine prothymocytes has been studied with an *in vitro* conversion assay. Several parameters of the assay have been examined closely in an effort to improve its reproducibility. These include methods used to isolate prothymocytes and the sensitivity of the cytotoxicity test used to detect the expressed antigen.

Glass and nylon wool columns as well as density centrifugation were used to separate prothymocytes from bone marrow and splenic populations. Nonadherent and low density fractions treated with thymic factor expressed Thy-1. Two to three times more Thy-1 positive cells could be detected with an improved counting method for the cytotoxicity test.

In addition, a new agent capable of inducing Thy-1 expression has been discovered. Sodium butyrate in low concentrations induces antigen expression on both bone marrow and splenic precursor T cells in a fashion similar to that of thymic factor induction.

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LITERATURE REVIEW

Thy-1 Antigen

Definition and distribution. In 1964, Reif and Allen (1) first demonstrated the presence of a shared allogeneic antigenic determinant in the thymocytes, peripheral T cells, and central nervous tissue of mice. It is now known that this alloantigen, Thy-1 (formerly known as theta antigen), is expressed as one of two allelic forms, Thy-1.1 (θ-AKR) or Thy-1.2 (θ-C3H). These are coded for by the Thy-1 locus on chromosome 9 of the mouse (2) and all mouse strains carry one or the other of these two antigenic specificities. It was originally thought that this antigenic system was species specific. However, the presence of a serologically indistinguishable antigen has also been described in rat brain and thymocytes (3). In contrast to the murine system, only one form of the antigen, Thy-1.1, has been demonstrated in rat tissue (3).

In the mouse, the membrane bound Thy-1 antigen has been used as a T cell marker and as an example of a differentiation antigen due to its distribution and the level of its appearance in murine tissue. It is found in high quantities only on immature T cells (thymocytes) and on brain tissue (1,4). This level appears to decline with T cell maturation, as Thy-1 is demonstrable only in small amounts on peripheral thymus-derived cells in mice (1). In addition, Thy-1 has been demonstrated on murine fibroblastic cell lines (5), epidermal cells (6), cells derived from mammary tissue (7), and cells in the peripheral and central nervous system (4). It has not been found on murine bone marrow or marrow derived lymphocytes (1), plasma cells (8) or granulocytes, macrophages, or red blood cells (9). In the rat, however, while

thymocyte and brain expression of Thy-1 are comparable to that in mice (10), it has been reported that as high as 30-40% of bone marrow cells express Thy-1.1 (11), and that rat peripheral T lymphocytes express little or no Thy-1 (10). Hunt et al. (12) reported that approximately 25% of the Thy-1 positive bone marrow cells were also positive for surface immunoglobulin, a B cell marker. They conclude that, in the rat, Thy-1 is not exclusively associated with thymic-dependent lineage.

Chemical characterization of Thy-1. The membrane bound nature of the Thy-1 molecule has posed some problems in its isolation and subsequent purification. Consequently, the exact chemical characterization of the antigen is still a matter of controversy. Thy-1 antigenicity has been proposed to be carried by a protein moiety (13), by a glycoprotein (14,15), or by glycolipids (16,17). This controversy may be due in part to the variety of methods used to isolate the Thy-1 molecule. It may also be related to the various assay procedures used to monitor Thy-1 activity during the isolation procedure. Detergent solubilization (18), immunoproecipitation (13), enzyme solubilization (19), and organic solvent extraction (20) have been the most widely employed isolation techniques.

Protein nature of Thy-1. Atwell et al. (13) described the isolation and partial characterization of a cell surface protein which carried Thy-1 determinants. Thymocytes were cell surface radioiodinated by lactoperoxidase; their proteins were extracted into organic solvents; and the antigen was identified by specific co-precipitation of radioactive cell surface proteins with anti-Thy-1 sera and rabbit antiserum to mouse immunoglobulin. Analysis of the immunoprecipitate by SDS-acrylamide disc electrophoresis led to the conclusion that Thy-1 was

a protein monomer of approximately 60,000 daltons. Other investigators (19), characterizing Thy-1 as expressed on a murine lymphoblastoid cell line, also reported that the Thy-1 antigen was associated with protein. Solubilization of the alloantigen was accomplished by limited papain digestion of the cells. Increased exposure of the papain digest to papain or to protease reduced the amount of antigenic activity as measured by a ^{51}Cr release cytotoxicity assay, suggesting that the Thy-1 determinant was protein associated. The size of the material possessing Thy-1 activity was estimated at 200,000 daltons by gel filtration of the concentrated papain digest on Sephadex G-200. On the other hand, Letarte-Muirhead et al. (18) demonstrated that Thy-1 activity isolated from rat tissue by detergent solubilization was associated with a globular protein of much smaller size, approximately 28,000 daltons. The molecular weight determination was based on gel filtration and sucrose gradient centrifugation of thymocyte membranes extracted with the detergents deoxycholate of Lubrol-PX. Thy-1 activity in the detergent extracts was monitored by inhibition of a radioactive binding assay specific for the alloantigen. Therefore, while all of these studies suggested that Thy-1 may be protein in nature, they did not agree on further physical parameters. Nor did any of them eliminate the possibility that the Thy-1 activity was associated with a carbohydrate portion of a glycoprotein or with glycolipids.

Glycoprotein nature of Thy-1. Trowbridge et al. (15) isolated a glycoprotein of about 25,000 daltons from mouse thymocyte membranes by immunoprecipitation. This molecule (T-25) was found on the surface of cell lines that carried Thy-1 antigen, but it was absent from derivative lines that lacked the antigen (21). Further studies by Letarte-Muirhead

et al. also described the Thy-1.1 alloantigen from rat thymocytes (14) and rat brain (22) as a glycoprotein weighing approximately 25,000 daltons. Their new purification procedure included not only detergent solubilization and gel filtration but also affinity chromatography on antibody or lentil lectin columns. Finally, Johnson et al. (23) reported that the Thy-1.2 alloantigen expressed on the S49 lymphoblastoid cell line was a glycoprotein.

Debate still exists, however, over which moiety of the Thy-1 glycoprotein is responsible for the antigenic activity of the molecule. The amino acid and carbohydrate composition of the purified rat Thy-1 antigen have been determined (24). The brain and thymus glycoproteins contained very similar amino acid compositions, but strikingly different carbohydrate moieties which accounted for approximately 30% of the molecular weight of the antigen. The authors, therefore, suggested a protein basis for the determinants since they could not detect any antigenic differences between brain and thymus Thy-1 (22). Furthermore, heating and proteolysis by pronase resulted in the loss of Thy-1 antigenic activity (24). However, it was also found that the antigenicity was not affected by other proteolytic enzymes, including trypsin and papain. No studies were done to alter the carbohydrate portion of the molecule. Others (21,23) have proposed that the antigenic determinants of the mouse Thy-1 molecule are on the carbohydrate portion of the glycoprotein. Johnson et al. (23) based their proposal on the fact that neuraminidase, which cleaves sialic acid from suitable substrates, was shown to cause the Thy-1 alloantigen from lymphoblastoid cell lines to lose its antigenic activity. It appears likely that protein may be only partially responsible for the molecule's antigenicity perhaps by serving

as a hapten carrier for the carbohydrate moiety.

Glycolipid nature of Thy-1. In their original description of Thy-1 antigen, Reif and Allen (4) demonstrated that the antigen was nondializable and sensitive to lipid solvents. Vittetta et al. (17) isolated a complex containing the Thy-1 antigen from the surface of murine thymocytes and T cells by cell surface radioiodination, lysis by freeze-thawing, and immunoprecipitation. This entity could be readily labeled with ^{3}H-galactose but not with labeled amino acids. It was also shown to have a lower density than protein. These observations prompted the authors to suggest that the antigenicity of the Thy-1 may reside in a glycolipid.

In accord with these findings, Esselman and Miller (20) proposed that mouse brain associated Thy-1 antigen was glycolipid in nature in view of their findings that the ganglioside rich upper layer of a Folch partition of brain or thymus tissue was capable of inhibiting the cytotoxicity of anti-brain associated Thy-1 antiserum when associated with auxiliary lipids. The isolated gangliosides G_{D1b} and G_{M1} possessed similar capacities and were, therefore, purported to carry Thy-1 antigenicity. Furthermore, pentasaccharides derived from these gangliosides were able to specifically inhibit the appropriate anti-Thy-1 sera in a hapten inhibition assay (25). Conversely, others (26) have shown by immunofluoresence that, while purified antibodies to G_{M1} did react with peripheral T cells and most thymocytes in several strains of mice, this reaction was independent of Thy-1 phenotype. More recent evidence suggests that the Thy-1 glycolipid was only a small part of the G_{M1} and G_{D1b} preparations used in earlier studies (27). These gangliosides had appeared to be pure in two distinct thin layer chromatography

systems. However, further fractionation by a third thin layer system resulted in the separation of a very minor component which contained all of the Thy-1 activity. An immune response assay was used to monitor the improved purification of Thy-1. Isolated Thy-1 glycoprotein reacted in parallel to the Thy-1 glycolipid in the assay. It was proposed, therefore, that the carbohydrate moiety of Thy-1 determined the antigenicity of the molecule and was conjugated either to a lipid or a protein carrier.

In contrast, Arndt et al. (28) found that extraction of murine thymocyte membranes with organic solvents yielded a considerable loss of activity of the Thy-1 antigen with some residual activity in the protein fraction but none in the organic phase. The reduced antigenic activity of the delipidated protein fraction could be restored by the addition of lipids. The investigators proposed that the thymocyte-brain antigen was protein in nature, but that lipid-protein interaction was necessary for the antigenicity of the Thy-1 molecule.

Further evidence that Thy-1 may be glycolipid in nature is the recent report by Thiele et al. (29) that choleragen and Thy-1 show a common ligand-induced redistribution when subjected to cocapping experiments. Since the ganglioside G_{M1} is the main receptor for cholera toxin (30), it appears likely that the Thy-1 antigen is either glycolipid in nature or closely associated with glycolipids in the membrane.

To date the exact chemical nature of the Thy-1 alloantigen is unknown. Evidence for both glycoprotein and glycolipid moieties has been shown. Its functional nature also is unknown. Thy-1 is shed from thymocytes (31) and lymphoblastoid cells in culture (32) suggesting that it is a peripheral rather than an integral member of the cell surface.

Proposals have been made that this shed material may play a nonspecific role in the modulation of the immune response (16). Purification of the antigen will aid in the discovery of its true biological role.

Thymic Factor

Introduction. The essential role of the thymus in the immune system was first demonstrated through the ability of thymic grafts to restore immunological competence to neonatally thymectomized (NTx) mice (33). Partial recovery of cell-mediated and humoral immune reactions was also observed when thymic grafts were enclosed in cell impermeable chambers (34,35) implying that a thymic humoral factor participated in the phenomenon. Subsequently, Trainin (36,37,38) reported that administration of thymus tissue extracts from different species to NTx mice partially restored their ability to produce immune responses to sheep red blood cells and to reject skin and tumor allografts. Preliminary experiments suggested that lymphoid cells, probably of thymus origin, were the site of action of these extracts (39). It was also suspected that these factors were produced by thymus epithelium (40).

Assays for thymic factor. A wide variety of methods have been used to isolate and assay a number of so-called thymic hormones. Not all of these methods were necessarily based on the restoration of immunocompetence. For example, G. Goldstein's thymopoietin is a purified polypeptide hormone isolated from bovine thymus and assayed by its effect on neuromuscular transmission in a laboratory model of the disease myasthenia gravis (41). Only later was it shown to be active in the induction of T-cell differentiation (42). A. Goldstein and White first tested the activity of their calf thymosin preparation with a "lymphopoietic" assay which measured DNA synthesis by lymph nodes after *in vivo* injection

of the extract (43). It was also discovered that thymosin was active in a bioassay based on the induction of azathrioprine sensitivity in rosette-forming spleen cells from adult thymectomized mice (44). Subsequently, J.F. Bach et al. discovered the presence of a substance in normal mouse serum which possessed thymosin-like activity in the rosette assay (45,46). This "circulating" thymic hormone was absent in the serum of thymectomized or nude mice suggesting that is was thymus specific. It was postulated that the capacity of the lymphoid cells to form rosettes correlated with restoration of immunocompetence and the ability of the cells to react with an antigen (47). Later, Komuro and Boyse found that thymosin was able to induce the development of cells bearing characteristic T lymphocyte surface antigens from populations of murine spleen or bone marrow cells (48,49).

Thy-1 induction assay. The study of T lymphocyte differentiation in the mouse has been aided by the presence of several distinctive surface antigens found on T lymphocytes in various differentiated states. These can be summarized as follows:

Antigens	Location
Thy-1 (θ)	Thymocytes, peripheral T cells and brain (1), epidermal cells (6), fibroblasts (5).
TL	Thymocytes only (50).
Ly 1, Ly 2,3	Thymocytes, peripheral T cells (51).
G_{1X}	Thymocytes and sperm of certain mouse strains (52).
MSLA	Thymocytes, peripheral T cells, brain, and epidermal cells (53).

Following a two hour incubation with thymosin, Komuro and Boyse (49) demonstrated the appearance of TL and Thy-1 antigens on approximately

20-30% of those bone marrow and spleen cells from normal mice which rested on the 10-23% interface of a discontinuous BSA gradient. They were also able to show the expression of TL and Thy-1 on embryonic liver cells and on bone marrow and spleen cells from 14 day old nu/nu mice (48). These findings demonstrated that the inducible cell had not undergone any previous thymus-mediated process. However, the actual mechanism of action of the relatively crude thymic extract which induced expression of the antigens was still a matter for speculation. The findings of the *in vivo* Thy-1 conversion assay implied that the inducible cell from bone marrow and spleen was committed to T cell differentiation even though it displayed none of the recognizable phenotypic traits of T lymphocytes.

Hormonal action of thymic factor. Several groups of investigators suggested that thymic factors may act in a hormonal fashion on the membrane of the precursor cell, perhaps through the intermediary second messenger cAMP. In support of this hypothesis Scheid et al. (54) demonstrated that agents of nonthymic origin, which had previously been shown to increase intracellular levels of cAMP in other systems, were also capable of inducing T cell surface antigens. These included Poly A:U, endotoxin, cAMP and DB-cAMP. In addition, insulin, a purported inhibitor of adenyl cyclase, decreased the expression of TL or Thy-1 antigens and aminophylline, a phosphodiesterase inhibitor, enhanced induction. It appeared, therefore, that any agent which increased cAMP levels was capable of inducing a predetermined Thy-1 negative precursor cell to become a Thy-1 positive cell.

Kook and Trainin (55) also demonstrated that agents which affect intracellular cAMP levels mimicked the activity of their thymus humoral factor (THF) as measured in an *in vitro* (graft vs. host) GVH response.

They also documented an increase in adenyl cyclase activity in spleen cells from neonatally thymectomized mice when they were incubated with THF for five minutes (56). Finally, Bach et al. (57,58) reported that low levels of cAMP could be substituted for their circulating TF *in vitro* to produce rosette forming cells. The effect of cAMP and thymic factor were found to be synergistic. They also found prostaglandins E_1 and E_2 as well as theophylline enhanced levels of rosette forming cells, while prostaglandins A_1 and A_2 and AMP had no effect (59).

Intracellular effects of thymic factor. The mode of action of thymic hormones, outside of a suspected involvement with the adenylate cyclase-cAMP system, remains undetermined. Initial experiments have shown that metabolic activity was required for TF action since no antigen expression can be demonstrated at temperatures less than or equal to $4^{o}C$ (32,42,49). Komuro and Boyse (49) demonstrated that cycloheximide could block the induction of Thy-1 expression on spleen cells suggesting that protein synthesis was required for hormone action. In experiments designed to test the induction of immunocompetence in lymphoid cells by THF, Kook and Trainin (56) also demonstrated the need for protein synthesis after studying the effects of cycloheximide. Two hypotheses were put forward by J.F. Bach to explain such evidence (59). First, thymic extracts induce the neosynthesis of Thy-1 antigen by activation of a gene that was not expressed previously. Second, these hormones elicit a membrane rearrangement which allows a sufficient amount of previously present antigen to become available for detection. Either gene activation or membrane rearrangement could be mediated by cAMP. The membrane rearrangement hypothesis was favored by Bach since Thy-1 induction *in vivo* by both TF (60) and cAMP (61) in adult thymectomized mice had been

shown to be reversible. Boyse and Abbot (62) postulated that the initial consequence of induction was the reconstitution of the cell surface by an earlier-expressed set of genes. Finally, Milewicz et al. (63) suggested that the modification of existing compounds on the cell's surface or the expression of a cryptic antigen accounted for the rapid rate of Thy-1 appearance (15 min) by TF rather than *de novo* synthesis.

In further experiments, Storrie et al. (64) have demonstrated that the induction of the surface phenotype requires both transcription and translation but not DNA replication. Metabolic inhibitors of RNA metabolism, actinomycin D, campothecin, and cordycepin, suppressed the expression of TL on precursor cells incubated with thymopoietin. These results indicated a need for newly synthesized RNA. It was suggested that this RNA could either specify the actual molecules that appear as cell surface components or that it could constitute or specify a regulatory molecule responsible for the display of preexisting components. Inhibitors of DNA replication, cytosine arabinoside and hydroxyurea, did not block induction of antigen expression. This finding was in accord with the rapid onset of the induction process ($\leq$ two hours) which does not appear to allow time for DNA replication. Definitive work on intracellular effects of TF will be possible when purer populations of precursor cells are obtainable.

Specificity of thymic factors. Extracts from different tissues prepared in parallel to thymic extracts were generally employed as controls during testing of thymic hormone activity. Occasionally, these supposedly inactive tissue extracts, for example, from calf muscle or spleen (54), would also induce antigen expression or mimic another

suspected property of thymic factors. During isolation of thymopoietin, G. Goldstein et al. (65) discovered another polypeptide of low molecular weight which was capable of inducing Thy-1 expression. This polypeptide was found in nearly every tissue tested. Consequently, it was named UBIP--ubiquitous immunopoietic polypeptide. Its presence in a wide variety of sources was thought to explain the occasional induction by tissues of non-thymic origin. Unlike low levels of thymopoietin, however, UBIP was not shown to be specific for T cell differentiation. UBIP also induced the appearance of complement receptors (CR) on precursor cells (66), which are purported B cell markers. Thymopoietin did not induce CR.

The specificity of thymopoietin was further shown by the effect of propanolol, a beta-adrenergic blocking agent, on TP or UBIP induced antigen expression (66). Precursor cells incubated with TP either in the absence or presence of propanolol still expressed TL but no CR. Conversely, cells incubated with UBIP in the presence of the agonist acquired neither TL or CR. These results suggested that TP and UBIP acted via two different receptors to induce antigen expression and that TP is the more likely physiological inducer. Since agents which elevate cAMP levels not only induce expression of T cell antigens but also purported B cell markers in a like fashion to UBIP, Scheid et al. (66) postulated further that these nonspecific inducers acted via the same adrenergic receptor as UBIP, not the supposed thymopoietin receptor.

In contrast to these findings, Hammerling et al. (67) have shown that TP at higher concentrations also affects mouse B cell differentiation by the induction of alloantigen Ia and surface Ig. Furthermore, striking effects were also demonstrated on granulocyte membranes (68).

Recently, Kagan et al. reported that complement receptors can be used as differentiation markers for granulocytes and that induction of this marker on precursor cells was demonstrated by both TP and ubiquitin (69). These authors have postulated that the specificity of TP resides not only in its selective inductive capacity on cells which possess its receptor, but also in the restricted distribution of the molecule *in vivo*. Therefore, TP may be a specific inducer of T cell differentiation *in vivo* only if it reaches an active concentration in the thymus.

Purified thymic hormones. Recently, much work has been done to purify to homogeneity the various factors described in thymic extracts and serum by different investigators. The amino acid sequence of three of these purified factors has now been reported. Dardenne et al. (70) sequentially purified and finally isolated the smallest of these factors, a circulating thymic factor (FTS), from 1000 liters of pig serum by ultra filtration, gel filtration, and ion exchange chromatography. The factor is a nonapeptide of sequence <Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn with a molecular weight of about 900 daltons (71). A synthetic peptide with the FTS sequence was produced by the Merrifield technique which displayed full biological activity and identical behavior to FTS on TLC and Sephadex G-25 (72). The properties possessed by FTS very closely resemble Trainin and Small's thymic humoral factor (THF) obtained from calf thymus (73). The amino acid sequence of a second polypeptide thymic hormone has been described by Schlesinger and G. Goldstein (74). Thymopoietin II is a larger (i.e. 49 amino acids) molecule with a molecular weight of 5562 daltons isolated from bovine thymus. These investigators were also able to synthesize an active peptide based on the determined amino acid sequence, but they recovered only 3% of the

activity of the parent polypeptide (75). Since the fragment had been based on an internal segment of the hormone, it was postulated that the tertiary structure of TP II may be required for optimal biological activity. This peptide seems to bear no relationship to FTS or THF based on amino acid sequencing. A. Goldstein et al. (76) have described a third sequenced factor, thymosin α_1. This was found to be one of approximately 10-15 major components found in the relatively crude thymosin fraction 5 isolated from calf thymus. This polypeptide is intermediate in length between FTS and TP II (i.e. 28 amino acid residues) and is heat stable and highly acidic. No sequence homology was demonstrable between thymosin α_1 and TP II, UBIP, FTS or THF. It was found that thymosin α_1 was more potent than fraction 5 in some T cell assays but not in others suggesting that more than one peptide component may be necessary to elicit full immunologic responsiveness. Whatever the case, it is apparent that a family of thymic factors exist, some of which appear to have overlapping functions. Perhaps, as suggested by Bach (71), more crude preparations, such as thymosin fraction 5, contain precursors of the smaller, more defined thymic factors. This hypothesis was suggested by a rise in serum FTS levels detected by the rosette assay after thymosin injection (77).

Sodium Butyrate

Butyrate treatment of cells. Short chain fatty acids and other lipophilic acids, some of which are used as antimicrobial food additives, inhibit bacterial growth and the uptake of amino and keto acids into bacterial membrane vesicles (78,79,80). Growth of certain mammalian cells in tissue culture also were inhibited by millimolar concentrations of sodium butyrate, a four carbon, short chain fatty acid (81).

At higher concentrations (10 mM and above), butyrate has been shown to be toxic to human cells in culture (82). More detailed studies of the effects of sodium butyrate were initiated after it was reported that the morphological and growth rate changes induced in tissue culture by dibutyrl-cAMP (DB-cAMP) could be mimicked by the control, sodium butyrate, alone (83).

Butyrate and cAMP. When DB-cAMP is added to the medium of certain cultured mammalian cells, the growth rate of the cells decreases and the morphology changes from a compact multi-layer to a monolayer of cells that appear to be more fibroblastic (83,85). Compounds which raise intracellular levels of cAMP, such as prostaglandin E_1, also elicit these growth changes (86). Wright (83), while studying the effect of DB-cAMP on morphology and growth rate of cultured Chinese hamster ovary (CHO) cells, discovered that 0.5 mM sodium butyrate produced similar results to those of the cyclic nucleotide. The changes were not identical since much longer incubation was needed in the presence of sodium butyrate to observe similar alterations in cellular morphology. The investigator speculated that either 1) the lipophilic fatty acid was affecting membrane bound adenyl cyclase thereby causing adjustments in cAMP levels; or 2) cell morphology, being dependent on the lipid content of the cell surface, was affected by the incorporation of sodium butyrate into the lipid component of the membrane.

Attempts have been made to measure directly the effects of butyrate on cAMP metabolism. In mouse neuroblastoma cells, addition of sodium butyrate elicited reversible inhibition of cell division but did not cause morphological differentiation as does DB-cAMP (87). It was shown in these cells that incubation with 0.5 mM sodium butyrate raised the

intracellular levels of cAMP two-fold (88,89). The basal level of adenyl cyclase activity in the mouse cells was also increased approximately two-fold (90). On the other hand, Tallman et al. (91) did not find increased adenylate cyclase activity, basal or fluoride activated, in sodium butyrate treated HeLa cells. They did report, however, increases in l-isoproterenol stimulated cyclase activity and cAMP accumulation, as well as increased synthesis of beta-adrenergic receptors.

Effects of sodium butyrate on other cell types showed a similar array of results (90). Low concentrations of butyrate induced similar morphological or growth rate changes in certain epithelial cell lines to those elicited in CHO cells. However, these epithelial cells were not inducible by exogenous cAMP (81,92). Butyrate, therefore, appears to act on mammalian cell cultures in at least two fashions. One mode mimics the action of increased intracellular levels of cAMP, and the other is unique to sodium butyrate itself.

Butyrate and glycolipid metabolism. Butyrate and other short chain fatty acids up to hexanoate induced filamentous protrusions in epithelial-like cell lines such as HeLa, Chang liver, L-132 and Intestine 407 (81). These morphological effects were not mediated or enhanced by DB-cAMP (81). In the course of examining the growth inhibition and marked morphological alteration induced in HeLa cells by 5 mM sodium butyrate, Fishman et al. (92) discovered a correlation between the morphology of HeLa cells in culture and their glycolipid content. In the presence of butyrate, HeLa cells contained 3.5 to 5 times more of the ganglioside G_{M3} than in its absence. This coincided with a 7-20 fold increase of the specific activity of CMP-sialic acid:lactosylceramide sialyltransferase within 24 hours of butyrate treatment (93,94). No

other glycosphingolipid enzyme or product was significantly altered by butyrate exposure. In addition, these intracellular glycolipid metabolic changes preceded any morphological alterations of the cell surface, prompting the suggestion of a possible role for glycolipids in the control of cell growth and structure (92).

The effects of butyrate treatment on glycolipid metabolism have been studied most extensively in HeLa cells. However, it has also been shown that treatment of neuroblastoma cells with butyrate resulted in a substantial change in ganglioside composition and an increase in two sialyltransferase activities (30). Moskal et al. (95) reported a similar increase in sialyltransferase activity in these cells treated with DB-cAMP. Furthermore, human epidermoid carcinoma (KB) cells have been examined for changes in glycosphingolipid metabolism in the presence of butyrate (96). KB cells exhibit characteristic process formation under butyrate treatment. Likewise, CMP-sialic acid:lactosylceramide sialytransferase was stimulated nine-fold. Using cell surface labeling techniques, the major glycolipid of the cell (GL-3) was increased two-fold on the surface of butyrate treated versus control cells. In addition, the activity of a specific galactosyl transferase was inhibited 50% after exposure to 2-4 mM butyrate for 15-24 hours. On the other hand, in Chinese hamster ovary cells, butyrate did not increase levels of G_{M3} ganglioside or its specific sialyltransferase (97). However, this may be because both are present normally at levels which are comparable to the induced levels measured in HeLa cells.

The manner in which butyrate-induced changes in ganglioside metabolism are related to morphological differentiation of cultured mammalian cells remains to be determined. Preliminary data from cell surface

labeling experiments indicated that approximately five-fold more G_{M3} was labeled in butyrate treated HeLa cells than in control cells (30). Fishman and Brady postulate, therefore, that newly synthesized ganglioside may be incorporated into the plasma membrane where it functions in a physical-chemical manner to promote extension of cell processes.

Molecular level of butyrate action. Butyrate has been shown to affect cAMP and glycolipid metabolism in certain mammalian cell cultures. Investigators have also examined the consequences of butyrate treatment on protein metabolism. In HeLa cells, process formation induced by butyrate was sensitive to inhibition by actinomycin D and cychoheximide (81,93). In addition, neurite extension was blocked by colcemid (93) and the calcium ionophore A23187 (97). These findings implied that butyrate required both protein synthesis and microtubule assembly to exert its morphological effects.

Nucleic acid metabolism has also been studied in mouse neuroblastoma cells incubated in the presence of 0.5 mM butyrate for three days. The DNA content per cell decreased by about one half (98) and total RNA and protein content increased 2-3 fold (99). In HeLa cells and chick embryo fibroblasts, DNA synthesis, as measured by uptake of ^{3}H-thymidine, was also strongly inhibited after butyrate exposure (100). In contrast to neuroblastoma cells, however, incorporation of labeled proline or uridine in HeLa cells showed that butyrate had little or no effect on overall RNA or protein content (100,101). It is not known whether these differences are caused by different assay methods to determine RNA and protein content, or by inherent differences in the cells themselves in their responses to butyrate treatment. During closer examination of neuroblastoma cells, it was found that, while the total

RNA content of butyrate treated cells increased, the concentration of poly A containing cytoplasmic RNA (presumed to be messenger RNA) did not change (99). Finally, in butyrate induced erythroid differentiation in cultured erythroleukemic cells, increased synthesis of a specific mRNA coding for globin has been demonstrated (102,103). It appears that careful examination of the different RNA types in these cells will be necessary before an overall hypothesis on the effect of butyrate on RNA metabolism can be stated.

An examination of the early time course of inhibition of DNA synthesis in HeLa cells and chick embryo fibroblasts demonstrated that after only one hour the rate of thymidine incorporation had fallen to 68% of the control (100). After 24 hours, DNA synthesis in butyrate treated cells was only 2-10% of control cells. Histone modification in both HeLa and Friend erythroleukemia cells has been shown to occur (100) by a time course which closely resembles the one of DNA synthesis inhibition. Acetylation of histone H4 can be demonstrated as early as one hour after the addition of butyrate to the medium (104). This has been shown to be due to an inhibition of histone deacetylase activity (105).

The thymidine incorporation experiments have led several investigators to postulate that butyrate treatment may led to cell synchrony. Hagopian et al. (100) suggest from the shape of the time course of inhibition of DNA synthesis and histone acetylation that cells in S phase during initial butyrate treatment are halted there and that others cannot enter it. Others (90,93,96) have found similar results and propose that butyrate may be synchronizing cells in the G1 phase of the cell cycle. The fact that butyrate arrests cells in G1 or S phase does

not entirely account for its action, however, since cells blocked in S phase by an alternate method do not display characteristic morphological or enzymatic alterations until butyrate is added (93). The fact that glycolipid biosynthesis has been shown to be maximal during G1 (106) may explain why glycosphingolipid levels appear to be so sensitive to butyrate treatment.

Specificity of butyrate. In Wright's original comparison of morphological changes induced by butyrate and DB-cAMP, he reported that sodium isobutyrate had no effect on the growth of CHO cells (83). Simmons et al. (93) found that C3, C4 or C5 saturated fatty acids produced typical process formation and caused enzyme induction in treated HeLa cells. Analogs of butyrate also were shown to have no effect on morphology. In addition, specificity has been demonstrated by Leder and Leder (102) in induction of erythroid differentiation in cultured erythroleukemic cells. They reported a requirement for a three carbon, straight chain, aliphatic residue attached to a carboxyl group. This requirement was based on the ineffectiveness of longer or shorter related fatty acids in eliciting differentiation and on the ineffectiveness of analogues such as isobutyramide, N-butyramine, butylacetate and 2-pentanone. Similar observations on the specificity of sodium butyrate induction of alkaline phosphatase activity in HeLa and other mammalian cells have been reported (107).

Target Cell for Inducing Agents

Stem Cells. Maximow postulated the existence of a pluripotent hemopoietic stem cell in 1909. Subsequently, controversy between proponents of monophyletic, dualistic and polyphyletic theories of blood cell differentiation existed for many years. It was not until

Till and McCulloch (108) described a technique for the detection of primitive haemopoietic cells present in low frequency in mouse spleen and bone marrow that the controversy abated. The cells were capable of forming macroscopically visible colonies of differentiating granulocytic, erythroid and megakaryocytic cells in spleens of lethally irradiated mice and were termed *in vivo* colony forming units (CFU). The clonal nature of these colonies was implied by the linearity of the plot relating the number of marrow cells injected to the number of colonies that developed in the spleen (100). More direct evidence was obtained with the use of radiation induced chromosomal markers (110,111). Wu et al. (111) reported that 91-100% of mitoses were found to be of unique karyotype in any one colony and that 10 out of 12 colonies showed the same unique marker for both erythroid or granulocytic metaphases. In light of such experiments, most investigators now agree the *in vivo* CFU is a multipotential stem cell (112).

Identification of CFU. Many attempts have been made to identify CFU's functionally and morphologically and an equal number of candidate stem cells have been proposed. The small lymphocytes of the bone marrow, as suggested by Cudkowicz et al. (113) have often been thought to fulfill this capacity, as have certain 'transitional lymphocytes' (114) described as large basophillic blast cells. Combining these theories, Yoffey (115) has postulated that no single specific stem cell exists, but rather that there is a compartment of cells which is in steady state. This lymphocyte transitional (LT) cell compartment is proposed to consist of 80-90% pachychromatic small lymphocytes and 10-20% leptochromatic transitional cells of various sizes and degrees of basophilia. Identification studies such as these have been hampered by the low numbers of

stem cells present in hemopoietic tissue. VanBekkum et al. (116) concentrated this population by repeated density centrifugation which was monitored by an *in vitro* assay for CFU's. The stem cell enumerated in this thin layer agar colony technique was termed a CFU-C and was thought to represent a similar cell to CFU (117). Both light and electron microscopy were used to describe the hemopoietic stem cell (HSC) in mice (116) and in human and monkey (118) bone marrow. The candidate HSC was shown to be quite distinct from small lymphocytes and close to Yoffey's lymphotransitional cell. While these investigators felt that they had identified the HSC, others criticized a strict morphological approach for identification. Experiments such as those of Worton et al. (119) supported the criticisms. In these studies, velocity sedimentation was used to separate both CFU-S and CFU-C populations according to size. Broad peaks were found for both groups indicating that the functionally homogeneous populations were heterogeneous in respect to size.

Relationship between CFU's and T lymphocytes. Much work has been done in an effort to delineate the relationship between CFU's and T lymphocytes. Originally, it had been postulated by Auerbach (120), that lymphoid cells in the thymus arose from the thymic epithelial compartment. However, it has since been shown that thymocytes develop from stem cells which migrate into the thymus from the yolk sac in embryonic chicks (121) and from the fetal liver in mice (122). Furthermore, in adult animals, chromosomal marker studies have demonstrated migration of stem cells between various hemopoietic organs, including traffic between bone marrow and thymus (123). Owen and Raff (124) reported that these cells appeared to be large blast-like cells which did not carry thymocyte alloantigens such as Thy-1 or TL on their surface. This implied that

they were undifferentiated precursors, perhaps CFU's. Indeed, the occurrence of spleen lymphoid colonies in the CFU assay was claimed by Mekori et al. (125) following the injection of large numbers of thymic or lymph node cells from phytohemagglutinin treated donors into irradiated recipient mice. However, others (126) could not duplicate these results and the claim was subsequently retracted. Since then, it has been postulated that lymphocytes do not form aggregates *in vivo* due to their migratory properties (127). This alone could account for the lack of lymphoid colonies in CFU assays.

Despite the failure of lymphoid colonies to occur, precursors of lymphoid populations have the same origin as CFU. Using radiation induced chromosomal markers to identify the progeny of singly hemopoietic stem cells, Wu et al. (128) found that cells with the same abnormal karyotype as CFU's in the spleen reached the thymus and lymph nodes of recipients within 1 month. Whether all CFU's are capable of differentiating into both myeloid and lymphoid cells, or whether CFU's and lymphocytes have a common precursor was not established.

Attempts to distinguish between CFU's and thymic factor sensitive cells have been made. Separation of bone marrow and spleen by density centrifugation has led to the finding that both CFU and prothymocyte concentrations are enriched in low density fractions of discontinuous BSA density gradients (49,129). However, unlike precursor T cells, CFU's have also been demonstrated in fractions of higher density (130). Density estimates for the low density CFU's isolated in such experiments vary slightly. El-Arini et al. (131) have reported that cells in the low density region of 1.050-1.059 gm/cm^3 become immunocompetent within 15 days after injection into the spleen of irradiated isogeneic

recipients. The results of Turner et al. (129), who estimate the density of the CFU's at around 1.051 gm/cm^3, corroborate that finding. Dicke et al. (118) found their HSC to migrate between the slightly higher density range of 1.055-1.0682 gm/cm^3. Some overlap was demonstrated when density estimates of the putative thymic precursor were reported. For example, Incefy et al. (132) found that the layer of cells settling on the 21-23% interface of a BSA gradient (density 1.0552-1.065 gm/cm^3) contained an enriched population of prothymocytes. Others have reported densities of 1.064 gm/cm^3 (131) and 1.068 gm/cm^3 for progenitor T cells (133). Therefore, while it appears that the precursor T cell may be slightly denser than a CFU, the minor differences in density distribution are not sufficient to distinguish between the two cell types.

Other evidence bearing on the relationship of CFU's to prothymocytes has been reported. Thymic precursor cells have been found to be capable of repopulating the thymus of a lethally irradiated mouse (134) as do CFU's. However, these repopulating cells have a different density from CFU's (131). Also, it has been shown that prothymocytes can be induced to express thymocyte differentiation antigens without influencing the number or distribution of CFU's (135). Taken together, the evidence suggests that the two cell populations are not the same.

Thymic precursor cells. Some cell surface characteristics of the suspected precursor T cell have been defined. They display H-2 antigen and at least two antigens recognized by antisera to mouse brain (133,136). Other investigators have reported that prothymocytes lack detectable amounts of surface markers associated with either B or T cells (133) placing them in the "null" cell population of the spleen (137). On the

other hand, Loor and Roelants (128) reported that, while a precursor T cell has no membrane Ig, it does possess a low density of Thy-1 antigen which is detectable only by indirect immunofluorescence. Their finding has been disputed in light of the multiple specificities of antisera used to detect Thy-1 (133).

Recently, the use of an internal cell marker for the identification of precursor T cells has been suggested (139). Terminal deoxyribonucleotidyl transferase (TdT) catalyzes the polymerization of deoxyribonucleotides in the presence of a primer without the requirement for a template (140). This enzyme is found uniquely associated with the thymus and bone marrow of mice (141). It can also be demonstrated in a developing chick embryo thymus (142). Pazmino et al. (143) found (TdT) only in the low density (10-23%) fraction of marrow separated by BSA density gradient. Furthermore, treatment of bone marrow cells with thymopoietin can make the TdT positive cells sensitive to anti-Thy-1 sera and complement (139). Both of these are properties which mimic those of prothymocytes. Silverstone et al. (139) postulate that TdT is the earliest known marker of cells that can undergo thymus dependent development. Others (144), however, have shown that TdT can also be demonstrated in the leukemic cells of a patient with B cell acute lymphocytic leukemia. These authors feel that TdT is related more to the immaturity and proliferation of certain lymphoid cells than to their progress toward B or T cell differentiation.

In erythropoiesis, it has been suggested that the erythropoietin sensitive cell (ESC) is already permanently committed to a restricted pathway of differentiation (145). Following irradiation, recovery rates of colony forming units differed from erythropoietic responsiveness.

Indirect evidence for pre-determined differentiation also exists for progenitor T cells. Scheid et al. (54) have demonstrated that a variety of non-thymic agents were capable of inducing T cell differentiation antigens on precursor cells. It has also been reported that thymic factors can induce T antigen expression on cells isolated from nu/nu mice which are genetically athymic and on cells from fetal liver (48,146). However, the fact that the presence of a thymus is not needed to induce differentiation is only a presumptive basis for predetermination. It remains possible that these cells are "proimmunocytes," rather than prothymocytes, capable of differentiation into either T or B cells depending on the environment to which they migrate (133). This might explain the existence of cells which express both B and T cell markers (147).

Stem Cell Separation Techniques

Introduction. Stem cells and prothymocytes have been isolated from two main sources in the mouse: the bone marrow and the spleen. These two hemopoietic organs contain a highly heterogeneous population of cells of both lymphoid and nonlymphoid origin. Furthermore, Dicke et al. (118) have estimated that the pluripotent hemopoietic stem cell is present at a concentration of only four to six per 1000 cells in bone marrow. This fact has hampered the development of definitive studies designed to morphologically and functionally identify stem cells and committed progenitor cells. To alleviate this problem, a variety of cell separation techniques have been applied to spleen and marrow suspensions. These fall into four main catagories, 1) filtration or adhesion to glass and nylon wools (148-151) columns, 2) isopycnic density gradient separation (49,63,115,152), 3) velocity sedimentation (153-155),

and 4) two dimensional cell separation (69,156,157).

Glass and nylon wool column separations. Glass wool or beads have been used to partition cell suspensions since it was reported that it was possible to purify peripheral blood leukocytes by allowing them to adhere to glass bottles (158). Cudkowicz et al. (159) demonstrated the partial purification of a suspected hemopoietic stem cell which resembled a small lymphocyte after the passage of mouse bone marrow through columns of glass wool. More recently, others (149) have used a similar technique to concentrate stem cell populations and have obtained a 5-fold increase in stem cells relative to unfiltered marrow. Progenitor T cells have also been found to be glass wool nonadherent (133) when spleen cells suspensions are passed over glass wool columns.

Further fractionation of lymphocytes and their precursors can be obtained if filtration over a glass wool column is followed by passage over a nylon wool column. Trizio and Cudkowicz (160) reported the separation of functional B and T cells from spleens with this double column technique. Both cell populations passed through glass wool columns while only T lymphocytes were found in the effluent fraction of nylon wool columns. Conflicting reports exist, however, on the adherence of precursor T cells to nylon wool. It was shown by Twomey et al. (151) that precursor T cells from spleen cell suspensions were eluted from nylon wool columns. Prothymocyte populations were demonstrated by the induction of Thy-1 antigen after incubation with thymopoietin and ubiquitin. In contrast, Basch and Kadish (133) found thymocyte precursors in both the adherent and nonadherent fractions after passage of splenocytes through nylon fibers. In this case, a quantitative assay which determined the number of donor type cells to repopulate the thymus of

an irradiated recipient was employed to monitor the presence of progenitor T cells (161). It is possible that the differences in assays systems employed may account for this apparent discrepancy or that subpopulations of precursor cells may exist.

The reason for the adherence of certain cell types to either glass or nylon wool remains unknown. It has been suggested that an active metabolic process is involved since cell separation values were low when columns were maintained and operated at 4°C rather than at room temperature or 37°C (149). In addition, siliconized glass bead columns separated cells by a temperature independent selective trapping by physical adherence or by size (162).

Isopycnic density gradient separation. The most commonly employed method to fractionate thymic precursor cells from heterogeneous hemopoietic populations has been isopycnic density gradient separation. In this technique, cells centrifuged in continuous or discontinuous density gradients are banded in locations in the gradient that are equal to their own densities. Previous to the description of a linear bovine albumin gradient by Leif and Vinograd (163), density separation and determination of cells had been done either by the packed cell method in which a cell pellet was separated by layers or by the neutral density method in which cells were centrifuged in a dense liquid medium and segregated into pelleted and floating fractions (164). Since the introduction of gradient density separation, however, these tedious methods have been abandoned.

Both stem cell (115) and prothymocytes (49) populations have been concentrated with the aid of discontinuous bovine serum albumin density gradients, and their suspected densities have been reported. These vary

from 1.051 gm/cm^3 for the hemopoietic stem cell (129) to 1.068 gm/cm^3 for a purported progenitor T cell (133).

A wide variety of gradient media have been examined and Ficoll and bovine serum albumin have been used most often. These substances leave the desired cell populations both viable and functional after separation. Recently, a new gradient medium has been described, metrizamide, which is a non-ionic, tri-iodinated benzamide derivative of glucose. This substance appears suitable for gradient separation of living, intact cells since it is possible to prepare isotonic, buffered metrizamide solutions of high density and low viscosity. It has been used successfully for blood cell separation (165), fractionation of rat liver cells (166), and separation of granulocyte-macrophage progenitor cells from mouse bone marrow (167).

Velocity sedimentation. Hemopoietic cell populations have also been fractionated by velocity sedimentation techniques. Miller and Phillips (168) developed a procedure in which a thin band of cells was allowed to sediment through a shallow gradient of 3-30% fetal calf serum at unit gravity. Velocity sedimentation such as this partitions cells on the basis of size, or volume, with only a small dependence on cell density.

Both stem cells and progenitor cells have been separated by velocity sedimentation. Worton et al. utilized this technique to demonstrate that stem cells differing in their self renewal capabilities could be fractionated on the basis of size (169). In contrast, others (118), have shown similar distribution profiles between CFU-S and CFU-C. Reasons for this difference may lie in differences in procedures.

Velocity sedimentation also possesses the capability of partitioning

progenitor cells of various classes. The thymocyte precursor cell has recently been examined by a modification of this technique (133) and has been shown to be similar in size to medium to large lymphocytes (i.e. 10-15 μm in diameter). Lafleur et al. (170) have found that B lymphocytes can be partitioned from their precursor cells by sedimentation at unit gravity. Finally, McCool (171) has been able to isolate erythropoietin sensitive cells from rat bone marrow with this procedure. Fractionation of cell suspensions by velocity sedimentation at unit gravity appears, therefore, to be a useful separation method.

Two dimensional cell separation. Several groups of investigators have combined two separation techniques based on distinct parameters in an effort to obtain more purified stem or progenitor cell populations. Haskill and Moore (157) employed both equilibrium density and velocity sedimentation to resolve differences between murine embryonic and adult hemopoietic stem cells. Also, Kagan et al. (69) separated human bone marrow first by density on Ficol-Hypaque gradients and then by size by velocity sedimentation to isolate a granulocyte progenitor population. In a third study, immature myeloid cells from human bone marrow were enriched by density and size as well as by hypotonic shock (to remove erythroid precursors) (156). After the final step of velocity sedimentation at unit gravity, fractions were collected that contained an average of 62.6% immature myeloid cells versus an average of 8.9% in the original unprocessed marrow. Finally, Paterson et al. (172) reported on the partial purification of lung mast cells after separations by both density and size on metrizamide gradients. Further examination of combination techniques should yield progressively purer populations of cell types from heterogeneous mixtures such as bone marrow and spleen.

Additional separation techniques. Several other cell separation methods have been employed which rely on cell surface properties directly. Immunoabsorbent column chromatography and rosette-depletion techniques have been used to eliminate cells with known B and T cell markers from peripheral blood mononuclear cells (173). This was done in an effort to concentrate the "null" cell population which has been thought to contain both stem and progenitor cells. Others (174) have identified mouse lymphocyte subpopulations by their natural binding of eight strains of bacteria. Differences in cell surface charges have been utilized to fractionate cell populations by electrophoresis (175) and by counter current distribution (176). Cells separated by such techniques remain both viable and functional. Finally, Herzenberg et al. (177) have utilized fluorescent labeled antibody to specific membrane receptors to partition cells with the aid of a fluorescence-activated cell sorter. This technique allows greater specificity than is afforded by physical criteria such as size and density alone.

INTRODUCTION TO EXPERIMENTAL REPORT

The ability of the thymus and thymic extracts to induce different stages of T cell differentiation has been documented by a variety of *in vivo* (34,36,37) and *in vitro* (44,48,55) assay systems. One of these assays, originally descirbed by Komuro and Boyse (48), monitors the *in vitro* expression of T cell specific differentiation antigens (Thy-1 and TL) on the surface of prothymocytes which are found in low numbers in the bone marrow and the spleen. Antigen appearance during incubation of precursor cells with thymic factor occurs in two hours or less, and a direct cytotoxicity test is employed to quantitate the percentage of cells induced to express the antigens. This is the earliest known effect of thymic factor on precursor T cells. Since the Thy-1 conversion assay appeared to be both rapid and precise, many investigators began to use it to assess tissue extracts and other compounds for thymic hormone activity (42,54,65). Others used it to examine the mode of action of thymic factor (63,64). Milewicz et al. (63) suggested that the rapid appearance of Thy-1 by thymic factor treatment was the result of the modification of existing membrane compounds or the expression of a cryptic antigen on the cell's surface. They also utilized the system to attempt to characterize the Thy-1 antigen chemically by comparing the expression of Thy-1 and G_{M1}, a ganglioside which carries the Thy-1 alloantigen, on the surface of the precursor cell.

Recently, it has been reported that difficulties exist with the reproduction of the Thy-1 conversion assay. Fournier and Bach (178) have found that spontaneous induction of Thy-1 antigen occurred in the absence of any inducing agent nearly 50% of the time after bovine serum albumin density gradient separation of splenocytes. Twomey et al. (151)

reported that a two hour incubation of splenocytes with thymic factor led to low or variable results. They found it necessary to employ an eighteen hour incubation of precursor cells with thymic hormone in order to obtain consistency.

Several parameters involved in the Thy-1 conversion assay have been examined in this report in an attempt to develop a more reproducible assay. The sensitivity of the cytotoxicity test used to detect the appearance of Thy-1 antigen on precursor cells has been studied. Also, this report examines methods used to separate the low numbers of precursor T cells from the heterogeneous population of hemopoietic cells which exist in the bone marrow and spleen. Finally, the discovery of a new agent capable of inducing Thy-1 antigen expression on precursor cells is reported here. Sodium butyrate at low concentrations is shown to induce antigen appearance in the conversion assay in a similar fashion to thymic factor.

MATERIALS AND METHODS

Cells. Inbred C3H/HeJ and AKR/J female mice (Jackson Laboratories, Bar Harbor, ME) and outbred ICR-Swiss female mice (Spartan Research Farms, Haslett, MI) were used at ages 8 to 20 weeks as donors of splenocytes, thymocytes, and bone marrow cells. Bone marrow was aspirated from the femurs and tibias of sacrificed animals into Minimal Essential Media (MEM-Grand Island Biological, Buffalo, NY) with the aid of a 25-gauge needle and further disaggregated by passage through a 27-gauge needle. After centrifugation, seven to ten mice depending upon their age and strain, would yield from 2-5 x 10^8 nucleated cells of 85% viability by the trypan blue exclusion method. Spleens or thymuses were gently teased with forceps into MEM. Single cell suspensions of splenocytes or thymocytes were obtained by passage through a 27-gauge needle. After washing by centrifugation, one spleen would yield approximately 1-2 x 10^8 nucleated cells of 85% viability dependent upon age and strain. Both the bone marrow cells and the splenocytes were then subjected to the various separation procedures described below.

Cell separation techniques. Populations of bone marrow cells or splenocytes were fractionated on the basis of two distinct parameters--either by their densities or by their ability to adhere to glass or nylon wool. Density fractionation was achieved by centrifugation of the cells on a discontinuous gradient of bovine serum albumin (BSA) as described by Milewicz et al. (63). BSA stock solutions were screened for endotoxin contamination by the Limulus lysate assay (179). This assay was kindly performed by Dr. Robert Moon of Michigan State University. The low density prothymocytes were found in equilibrium with the 21% and 23% layers of a 17-27% step gradient. These were washed twice and then

pooled for direct use in the conversion assay described below. Upon examination by light microscopy, cells from the pooled fractions were found to be primarily mononuclear and to have a viability of greater than 95%. A small degree of contamination by polymorphonuclear cells and erythrocytes was present. The latter could be eliminated by subjecting the cell suspension to cold hypotonic shock with a NH_4Cl-EDTA-HEPES buffer (0.155 M, 0.1 mM, 1.0 mM, pH 7.35) for five minutes followed by several washes with fresh medium (180). The pooled 21% and 23% layers accounted for less than 10% of the total population of cells applied to the gradient. When tested with the appropriate antiserum and complement, these layers from bone marrow suspensions were found to be only 1-2% Thy-1 positive, and those from the spleen were less than 5% positive.

Fractionation based on adherence properties essentially followed the procedure developed for the separation of B and T lymphocytes (160). The only major exception to the originally described procedure was the substitution of BSA (2.5 mg/ml) for the 10% fetal calf serum in the incubation and elution media. Cells were first passed over a loosely packed glass wool (Corning Glass Works, Corning, NY) column, pelleted, and subjected to cold hypotonic shock. The remaining cells were then incubated on nylon wool (Fenwal Laboratories, Morton Grove, IL) columns for 45 minutes at 37°C. Nonadherent and adherent cells were collected. The effluent fraction, which accounted for 5-6% of the bone marrow cells and 10-15% of the splenocytes applied to the nylon column, consisted of primarily mononuclear cells with a few polymorphonuclear cells. The nylon wool adherent fraction contained all major cell types of the original suspension. Both fractions had viabilities of greater than 80%.

In vitro Thy-1 conversion assay. Marrow and spleen cells (10^6 cells/ml) separated by the above procedures were incubated for two hours at 37°C, 5% CO_2 in the presence of either thymic factor (15-90 µg/ml) as previously described (181) or in the presence of sodium butyrate (2 mM) to induce the expression of Thy-1 antigen. Thymic factor (TF), fraction 3, was prepared from fresh calf thymus according to Hooper et al. (182) and was used at the concentration of 90 µg protein/ml. Commercially prepared TF, fraction 5 (Hoffman-Laroche Inc., Nutley, NJ) was utilized at 15 µg protein/ml. Stock sodium butyrate (100 mM, pH 7.0) was prepared by the addition of sodium hydroxide to butyric acid (Sigma, St. Louis, MO). BSA (20 µg/ml) was used as a control. Following incubation, the cell suspensions were pelleted by centrifugation at 1600 rpm for 10 minutes, washed once, and resuspended in fresh medium at a concentration of 2.5×10^6 cells/ml for use in the cytotoxicity test.

Detection of expressed Thy-1 antigen - Antisera and complement. AKR anti-C3H (anti-Thy-1.2) and C3H anti-AKR (anti-Thy-1.1) antisera were prepared according to Reif and Allen (1). Titers of these sera were approximately 3200 and 1500 respectively. Normal AKR and C3H sera were used as controls. Guinea pig complement (Grand Island Biological, Buffalo, NY) was adsorbed with agarose and stored in aliquots at -70°C dilutes 1:3 with MEM.

Cytotoxicity test. Treated bone marrow and spleen cells were tested for the expression of Thy-1 antigen in a direct cytotoxicity test patterned after that of Boyse (183) which used the uptake of trypan blue as a measure of cell death. 10^6 washed cells were suspended in 360 µl of MEM. To this was added 20 µl of the appropriate antisera (final dilution

1/2000) and 20 µl of complement (final dilution 1/60). Complement or normal sera controls were adjusted with MEM to obtain the same final volume. The samples were then incubated for 45 minutes at 37°C, 5% CO_2 following which trypan blue was added to a final dilution of 0.02%. Both live and dead (i.e. blue) cells in a constant volume were counted in an eosinophil counter. The percent of conversion to Thy-1 positive cells was then calculated by two methods.

(1) % Thy-1 positive cells = % cells dead with anti-Thy-1 sera - % cells dead with control sera

(2) % Thy-1 positive cells = $\frac{\text{\# viable cells in control} - \text{\# viable cells in anti-Thy-1 sample}}{\text{\# viable cells in control}} \times 100$

The first method was based on a comparison of the percent dead in the control and anti-Thy-1 treated samples. The second method utilized absolute numbers of only viable cells to calculate percent conversion to Thy-1 positive cells. This approach accounted not only for dead cells which have taken up the trypan blue dye but also for cells which had undergone total lysis following antiserum and complement treatment (184). Calculations based on the first method are referred to as % Thy-1 positive cells by change in % dead. Those based on the second formula are referred to as % Thy-1 positive cells by change in number viable. All samples have been done in duplicate or triplicate and are presented as means with standard errors.

Absorption of anti-Thy-1 sera. Absorption of anti-Thy-1.2 sera was patterned after Basch and Goldstein (42). A dilution of anti-Thy-1.2 sera that gave approximately 50% cytotoxicity with C3H thymocytes was absorbed overnite at 4°C with various dilutions of butyrate or TF treated ICR-Swiss treated bone marrow cells (ranging from 2×10^6 to 2.5×10^5)

pooled from 21% and 23% layers of BSA density gradient. The serum was centrifuged free of cells and tested for residual cytotoxicity for C3H thymocytes. Equivalent numbers of BSA treated bone marrow cells were used as a control.

RESULTS

Sensitivity of the Cytotoxicity Test

Increased detection of Thy-1 inducible bone marrow cells. Bone marrow cells, pooled from the 21% and 23% layers of a discontinuous BSA density gradient, were treated with TF for two hours to induce the expression of Thy-1. The newly expressed antigen was then detected by a direct cytotoxicity test. The sensitivity of this test could be manipulated by changing the procedure for counting viable and dead cells after the addition of trypan blue. These results are shown in Table I. The percentage of cells induced to express Thy-1 was obtained formerly by subtracting the percentage of cells killed by control sera from the percentage of cells killed by anti-Thy-1 sera (formula 1 in Materials and Methods--% Thy-1 positive cells by change in % dead). If care was taken to insure that each sample being analyzed contained the same concentration of cells at the start of the cytotoxicity test, the percentage of cells induced to express antigen could also be calculated in a more precise manner. This method involved counting only viable cells in a set number of chambers in an eosinophil counter. The number of dead cells was obtained arithmetically by subtracting the number of viable cells in the anti-Thy-1 treated sample from the number of viable cells in the control sample (formula 2 in Materials and Methods--% Thy-1 positive cells by change in number viable). As seen in Table I, the percentages of Thy-1 induced cells based on viable cell counts were found to be consistently two to three times greater than those based on a comparison of death percentages. The increased detection of Thy-1 positive cells by viability counting was probably due to the fact that some lysed cells, which were previously lost from the counting because of their presence

TABLE I

INDUCTION OF THY-1.1 AND THY-1.2 ON MURINE

BONE MARROW CELLS BY THYMIC FACTOR

STRAIN	TREATMENT[c]	ANTISERUM	% DEAD	% THY-1 POSITIVE[d] (Δ % DEAD)	VIABLE[e]	% THY-1 POSITIVE[d] (Δ NUMBER VIABLE)
AKR[a] (THY-1.1)	BSA	-	16		637 ± 1	
				1		0
		ANTI-THY-1.1	17		635 ± 20	
	TF	-	17		611 ± 40	
				7		14
		ANTI-THY-1.1	24		528 ± 37	
C3H[b] (THY-1.2)	BSA	-	20		353 ± 15	
				1		0
		ANTI-THY-1.2	21		353 ± 18	
	TF	-	20		376 ± 16	
				8		22
		ANTI-THY-1.2	28		293 ± 39	

[a]Cells obtained from pooled 21% and 23% layers of BSA density gradient.

[b]Cells obtained from pooled 21% and 23% layers of BSA density gradient followed by cold hypotonic shock and washed twice prior to TF incubation.

[c]10^6 cells/ml were incubated for 2 hours at 35°C, 5% CO_2 with TF (15-90 µg protein/ml) or BSA (20 µg/ml) as a control. Cells were washed twice prior to cytotoxicity test.

[d]Percentages calculated from formulas presented in Materials and Methods.

[e]Average and standard error from duplicate samples.

only as debris, were included by this method. This phenomenon was not due to increased cell fragility caused by the hypotonic shock treatment used to eliminate contaminating erythrocytes since it occured with both shocked (C3H) and nonshocked (AKR) cells (Table I). To insure that the observed differences were significant, large numbers of viable cells (normally 300 to 600 cells for each sample) were counted. Only small standard errors were found between duplicate samples (generally less than 10%). In addition, death percentages for both anti-Thy-1 treated and control samples were always calculated to validate duplicates. Such precautions were necessary to accurately quantitate the relatively small (15-20%) loss of viable cells to death and total lysis.

Detection of Thy-1 inducible splenocytes. In addition to their presence in bone marrow, precursor T cells have also been found in the adult mouse's other major hemopoietic organ, the spleen. As with bone marrow cells, incubation with TF induced the expression of Thy-1 antigen on approximately 15-20% of the low density fraction of BSA density gradient separated splenocytes (Table II). However, unlike the case with bone marrow percentages, the splenic percentage of Thy-1 inducible cells was demonstrable by either of the two counting methods employed regardless of the level of sensitivity. Since the assay systems used to induce Thy-1 on the two cell types were identical, it is probable that some difference may exist within the cells themselves which makes the precursor splenocytes less susceptible to total lysis following anti-Thy-1 and complement treatment. A basis for this difference remains to be determined.

TABLE II

INDUCTION OF THY-1.2 CELLS ON

C^3H SPLENOCYTES BY THYMIC FACTOR

TREATMENT[a]	ANTI-THY-1.2	% DEAD	% THY-1 POSITIVE[b] (Δ % DEAD)	VIABLE[c]	% THY-1 POSITIVE[b] (Δ NUMBER VIABLE)
TF	-	33	17	252 ± 9	17
	+	50		208 ± 7	

[a]Splenocytes were obtained from pooled 21% and 23% layers of BSA density gradient and found to be less than 5% Thy-1.2 positive when teated with anti-Thy-1.2 sera and complement. TF induction performed as described in Table I.

[b]Percentage calculated from formulas presented in Materials and Methods.

[c]Average and standard error of 3-4 samples.

Sodium Butyrate Induction of Thy-1 Expression

Sodium butyrate at the concentration of 2 mM induced the expression of Thy-1 antigen in the low density fractions of BSA gradient separated bone marrow cells of mice (Table III). Butyrate treatment induced antigen appearance only in cells of the 21% and 23% layers of the gradient (results not shown) as does TF treatment (63). Both Thy-1.1 and Thy-1.2 were shown to be inducible by butyrate. When the two different counting methods described above were employed to detect the number of Thy-1 inducible cells, the same increase in sensitivity was observed with the use of viability counting as had been observed with TF treatment. Further similarities between TF and butyrate induction of antigen expression are shown in Table IV. Approximately the same percentage (15-20%) of bone marrow cells became Thy-1 positive after butyrate as after TF treatment. In addition, butyrate was capable of inducing Thy-1 appearance in low density splenocytes as well as low density marrow cells.

Absorption of Anti-Thy-1 Sera by TF or Butyrate Treated Marrow Cells

It is possible that TF or butyrate treatment of marrow cells may cause an artificial increase in the number of cells thought to be Thy-1 positive due to the exposure of additional complement receptors or to the uncovering of autologous antigens or other nonspecific determinants. Both TF and butyrate treated cells, however, were shown to have an absolute increase in the amount of Thy-1 present on their surfaces by their ability to absorb the activity of anti-Thy-1 sera (Figure 1). Increasing numbers of TF or butyrate treated marrow cells absorbed increasing quantities of anti-Thy-1.2 antibodies from the sera, while control cells treated with BSA had no capacity for absorption at any concentration. In addition, the fact that TF and butyrate treated cells absorbed

TABLE III

INDUCTION OF THY-1.1 AND THY-1.2 ON MURINE

BONE MARROW CELLS BY SODIUM BUTYRATE

STRAIN[a]	TREATMENT[b]	ANTISERUM	% DEAD	% THY-1 POSITIVE[c] (Δ % DEAD)	VIABLE[d]	% THY-1 POSITIVE[c] (Δ NUMBER VIABLE)
AKR (THY-1.1)	BSA	-	16	1	637 ± 1	0
		ANTI-THY-1.1	17		635 ± 20	
	BUTYRATE	-	17	7	518 ± 39	15
		ANTI-THY-1.1	24		450 ± 18	
ICR-SWISS (THY-1.2)	BSA	-	12	3	279 ± 19	0
		ANTI-THY-1.2	15		312 ± 5	
	BUTYRATE	-	14	11	279 ± 23	25
		ANTI-THY-1.2	25		209 ± 15	

[a] Cells obtained from pooled 21% and 23% layers of BSA density gradient.

[b] 10^6 cells/ml were incubated for two hours at 37°C, 5% CO_2 with sodium butyrate (2 mM) or BSA (20 µg/ml) as a control. Cells were washed twice prior to cytotoxicity test.

[c] Percentages calculated from formulas presented in Materials and Methods.

[d] Average and standard error of duplicate samples.

TABLE IV

INDUCTION OF THY-1 POSITIVE CELLS IN MURINE BONE MARROW AND SPLEEN CELLS FRACTIONATED BY BSA DENSITY GRADIENT CENTRIFUGATION

CELL SOURCE[a]	STRAIN	TREATMENT[b]	% THY-1 POSITIVE[c] (Δ % DEAD)	% THY-1 POSITIVE[c] (Δ NUMBER VIABLE)	NUMBER OF EXPERIMENTS
Bone Marrow	ICR-Swiss	BSA	<1	3	7
	(1.2)	TF	7	22	10
		Butyrate	7	15	7
	C3H	BSA	<1	0	3
	(1.2)	TF	7	17	3
		Butyrate	ND[d]	ND	ND
	AKR	BSA	0	0	3
	(1.1)	TF	10	19	3
		Butyrate	11	21	5
Spleen	C3H	BSA	4	<1	4
	(1.2)	TF	15	10	4
		Butyrate	15	19	3

[a]Cells pooled from 21% - 23% layers of BSA density gradient.

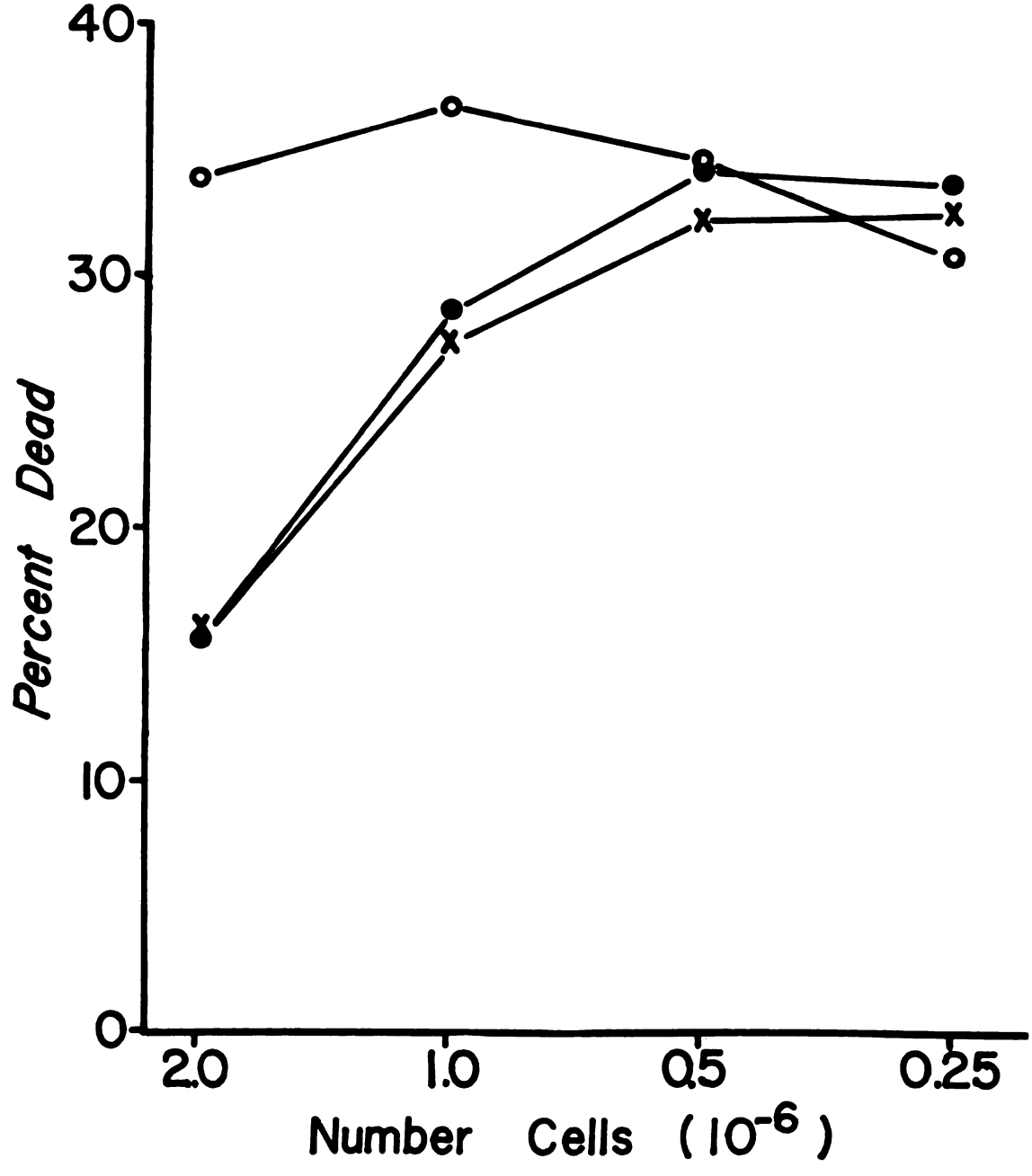
[b]Treatment as described in Tables I and III.

[c]Percentages calculated from formulas presented in Materials and Methods.

[d]Not done.

FIGURE I

Absorption of anti-Thy-1.2 serum with TF and butyrate treated bone marrow cells. ICR-Swiss bone marrow cells used for absorption were obtained from pooled 21% and 23% layers of BSA density gradients. Anti-Thy-1.2 sera of 50% cytotoxicity against C3H thymocytes was absorbed overnite with BSA treated (O), TF treated (●), or butyrate treated (X) marrow cells at varying concentrations and tested for residual cytotoxicity. Each point is an average of two experiments. Calculations are based on method 1 described in Materials and Methods. Further details in Materials and Methods.


40
30
20
10
0
Percent Dead
2.0
1.0
0.5
0.25
Number Cells (10^{-6})

anti-Thy-1 sera in nearly identical patterns is further evidence that butyrate induction of antigen expression is similar to that of TF.

Prothymocyte Separation - The use of Glass and Nylon Wool Columns

It has been suggested that inconsistent results in the *in vitro* conversion assay may be due to increased cell fragility caused by BSA fractionation and prolonged incubation with inducing agents (178). We have documented an increase in the amount of cell lysis with TF and butrate treated bone marrow cells. To test the hypothesis that this is due to BSA fractionation, an alternative method of cell separation was chosen--adherence to glass and nylon wool columns. The standard separation technique was modified to use only low levels of BSA (2.5 mg/ml) in place of the 10% fetal calf serum. It was also hoped that this method might improve on the purification of stem cells obtained by density centrifugation. Results are shown in Table V. Both the nylon wool adherent and nonadherent fractions were found to contain all Thy-1 negative cells prior to TF treatment (results not shown). After treatment, the effluent fraction had 20% Thy-1 positive cells while the retained fraction still had none. Enrichment of stem cells in the nylon wool eluted fraction was, therefore, comparable to, but no greater than, the low density fractions of a BSA gradient. It appeared that substituting low levels of BSA had no effect on the degree of cell fragility. Conversion percentages obtained with viability counting were still twice those calculated with death percentages indicating that the same ratio of cells expressing Thy-1 were being subjected to lysis and disintegration in a two hour period.

Similar results were obtained when splenocytes were separated by glass and nylon wool columns (Table VI). The inducible cell population

TABLE V

INDUCTION OF THY-1.2 POSITIVE CELLS BY THYMIC FACTOR IN C3H BONE MARROW FRACTIONATED BY GLASS AND NYLON WOOL COLUMNS

CELLS[a]	TREATMENT[b]	% THY-1 POSITIVE[c] (Δ % DEAD)	% THY-1 POSITIVE[c] (Δ NUMBER VIABLE)
Nylon Wool Eluted	TF	9	20
Nylon Wool Retained	TF	0	0

[a]See Materials and Methods for details.

[b]Treatment as described in Table I.

[c]Average six experiments - Percentages calculated from formulas in Materials and Methods.

[d]Average Two experiments - Percentages calculated from formulas in Materials and Methods.

TABLE VI

DETECTION OF THY-1.2 POSITIVE CELLS IN C3H SPLENOCYTES FRACTIONATED BY GLASS AND NYLON WOOL COLUMNS AND TREATED WITH THYMIC FACTOR

CELLS[a]	TREATMENT[b]	% THY-1 POSITIVE (% DEAD)[c]	% THY-1 POSITIVE (% VIABLE)[c]	CONVERSION Δ % DEAD	CONVERSION Δ % VIABLE
Whole Spleen	BSA	16	19		
				0	0
	TF	14	13		
Nylon Wool Eluted	BSA	18	20		
				11	27
	TF	29	47		
Nylon Wool Retained	BSA	0	3		
				4	<0
	TF	4	0		

[a] See Materials and Methods for details.

[b] Treatment as described in Table I.

[c] Average of triplicate samples - Percentages calculated from formulas presented in Materials and Methods.

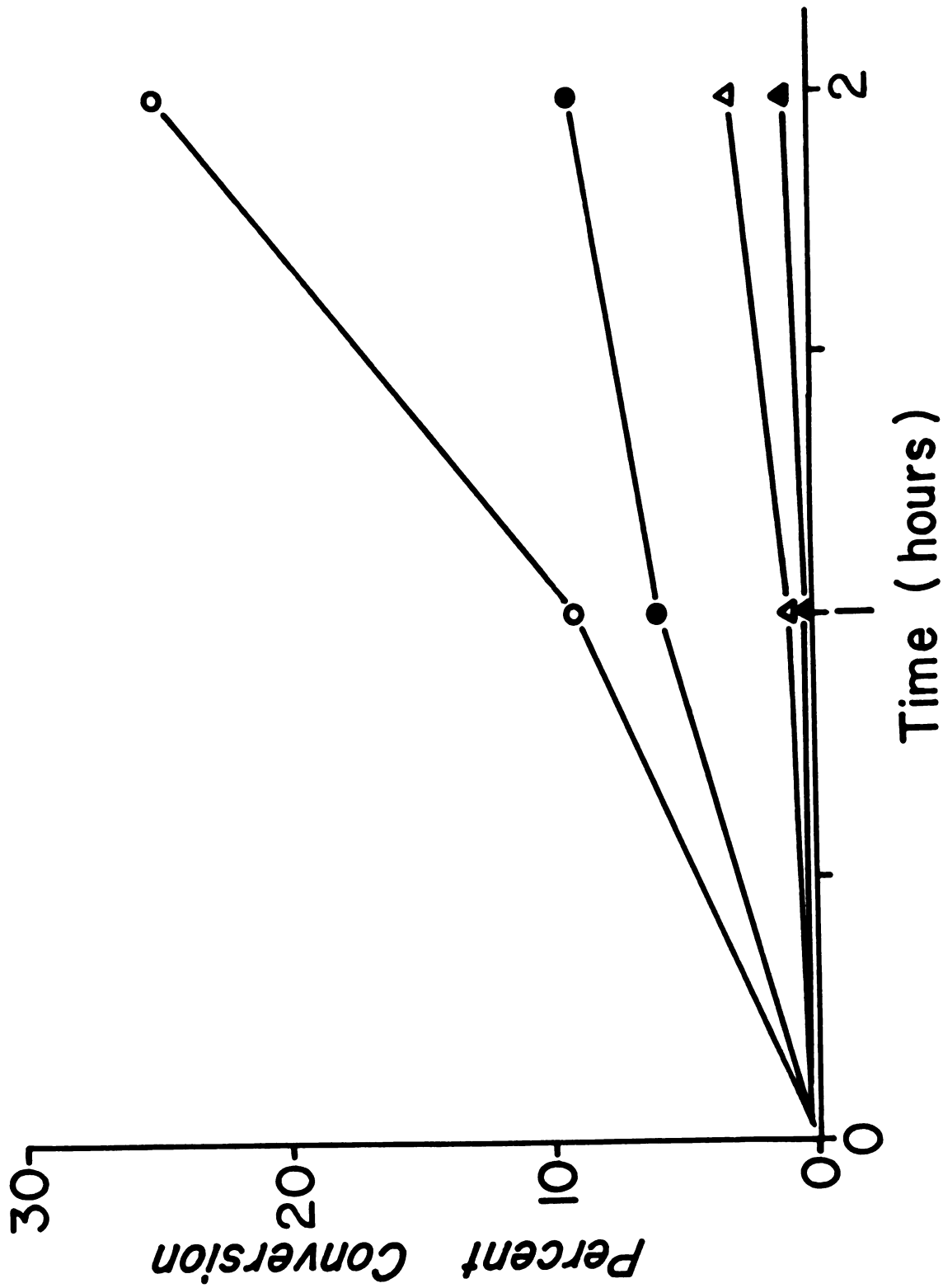
was found in the nylon wool nonadherent fraction (nearly 30%). This population was discovered over a background of Thy-1 positive cells (approximately 20%) that normally reside in the spleen and which were also nonadherent to nylon wool. The cells which were retained by the column were neither Thy-1 positive before nor after TF treatment. As with bone marrow cells separated by the columns, inducible splenocytes showed the characteristic increase in detection of Thy-1 positive cells when the more sensitive counting procedure was used. This finding, in conjunction with the results shown in Table II, suggest that, instead of increasing fragility, BSA fractionation may stabilize splenocytes in some cases.

Time Course of Increased Cell Lysis

The suggestion that prolonged incubation times for the *in vitro* conversion to Thy-1 positive cells may cause increased cell fragility (178) was also examined. Bone marrow cells separated by glass and nylon wool columns were incubated with TF for various times before being tested for the presence of Thy-1. It was found that after one hour of incubation the percentages of cells found to be Thy-1 positive were essentially the same regardless of the method used to calculate them (Figure 2). Past one hour, an increase in the number of Thy-1 positive cells was not detectable unless calculations were being made on viable cell counts. It is possible, therefore, that the length of the induction assay may have an effect on the number of Thy-1 positive cells which can be quantitated by the traditional method of counting dead cells in a trypan blue exclusion cytotoxicity test.

FIGURE II

Time course of increased cell lysis during the induction of Thy-1 on glass and nylon wool separated bone marrow cells. Nylon wool eluted and retained fractions were treated with TF for various lengths of time and tested for the presence of Thy-1. Percentages are based on formulas presented in Materials and Methods. Open symbols (O, Δ) refer to percent Thy-1 positive cells by change in number viable. Closed symbols (●, ▲) refer to percent Thy-1 positive cells by change in percent dead. Circles (O, ●) refer to nylon wool eluted fractions. Triangles (Δ, ▲) refer to nylon wool retained fractions.


Percent Conversion
30
20
10
0
0
1
2
Time (hours)

DISCUSSION

A rapid, precise, and reproducible assay for thymic hormones would greatly aid the study of T cell differentiation. The Komuro and Boyse Thy-1 conversion assay (48), which measures the appearance of T cell specific antigens on precursor T cells, potentially is such an assay but, practically, often falls short of the description. Some of the inherent difficulties of the system have been examined in this report. These include the sensitivity and reproducibility of the cytotoxicity test used to detect the expressed antigen, the heterogeneity in the agents used to induce antigen expression, and the small number of precursor cells present in the highly heterogeneous hemopoietic organs and the methods used to isolate them.

Others have attempted to improve the sensitivity of the cytotoxicity test. Twomney et al. (151), for example, added a proteolytic enzyme to the final incubation mixture in an attempt to detect Thy-1 positive cells which had suffered only a small degree of membrane damage from antibody and complement treatment. They still found it necessary, however, to extend their TF incubation time considerably beyond two hours to obtain consistent results. On the other hand, it has been shown here that, instead of adding another variable to an already complex system, a simple change in counting procedures could increase the sensitivity of the test. In some instances, as much as a three-fold increase in the detection of Thy-1 inducible cells could be demonstrated (Tables I and III). It is probable that this increase is due to the fact that the viability counting method accounts for all cells killed by anti-Thy-1 sera and complement regardless of the stage of cellular damage and disintegration. This suspected increase in the number of lysed cells has

not been previously documented in the Thy-1 conversion system, but other investigators (178) have reported that inducing agents occasionally lead to spurious results. Perhaps these inconsistent findings have been due in part to the loss of some Thy-1 positive cells to total lysis, and viability counting would have improved consistency. Attempts have been made to identify morphologically the cell population which is lysed (results not shown). Light microscopy revealed few differences. Wright stained smears of treated samples from the 21% and 23% layers of the BSA gradient appeared morphologically homogeneous even though it is apparent that these cells are functionally heterogeneous.

The actual reason for the increase in the number of Thy-1 positive cells susceptible to disintegration remains to be determined. One possibility is that *in vitro* handling of the cells may change their fragility. It has been suggested that BSA gradient separation of prothymocytes might significantly alter their fragility rendering the cells sensitive to anti-Thy-1 sera even though they have only minute quantities of the antigen on their surfaces (185). Two different methods of cell separation have been used here to examine this possibility, one of which (column separation) uses only low levels of BSA. Virtually no difference was found between the two techniques in the ratio of bone marrow cells susceptible to lysis (Tables IV and V). In the case of inducible splenocytes. it was shown that BSA gradient separation may actually stabilize the cells rather than increase their fragility (Tables II and VI). It appears, therefore, that high levels of BSA do not alone account for altered fragility of precursor cells. The significance of the difference between bone marrow and spleen inducible cells is unknown. In theory, the *in vitro* Thy-1 conversion assay tests for the same

precursor cell regardless of its location in the body. However, until this cell is isolated, or at least separated into a more homogeneous population, it will be difficult to accurately assess any other than gross morphological or functional differences between candidate prothymocytes of various sources. Perhaps precursors of different subpopulations of T cells exist in different organs.

Another *in vitro* condition which may affect cell fragility is the actual length of the induction assay itself. The length of incubation time with various inducing agents has been suggested by Bach (178) to affect the ability of a given method to detect antigen expression. Bach felt that his rosette-forming assay may have been more reliable than the Thy-1 induction assay partially due to the shorter time span needed to quantitate the antigen. It has been shown here that the prothymocyte's susceptibility to total lysis does appear to increase under lengthened assay conditions (Figure 2). Therefore, if standard counting methods were employed, the reliability of the *in vitro* conversion assay would drop after a sixty minute incubation period. Viability counting has been shown here to restore lost sensitivity. On the other hand, some feel that more consistent results may be attained with longer (i.e. 18 hour) rather than shorter incubations (151). It has been suggested that stem cells are present in the bone marrow and spleen in various transitional states correlating with their stage in the cell cycle (115). If this were true, additional precursors would continually enter the inducible cell cycle stage during a prolonged assay. The experiments documented in this report have not been extended past two hours. This was done to avoid further complications brought on by long incubations since these experiments were originally designed to study only the

initial consequences of TF treatment. Therefore, it is not known whether the total number of Thy-1 converted cells plateaued at 20-30% of the low density or nonadherent fractions, or whether it was actually higher.

Finally, increased disintegration of Thy-1 positive bone marrow cells may not be due to increased cell fragility but rather to the abundant amount of Thy-1 antigen which is induced on these cells. TF treated bone marrow cells (in an 18 hour assay) have been shown to have 6-12 times more Thy-1 expressed on their surfaces than the average amount of Thy-1 on normal thymocytes (42). A highly cytotoxic anti-Thy-1 sera such as the ones used here, therefore, would be capable of sensitizing a cell sufficiently to cause extensive damage upon the addition of complement.

Other factors may influence the reproducibility of the Thy-1 induction assay. The presence of contaminating endotoxin in the BSA gradient used for cell separation is another possible reason for inconsistent results. Endotoxin has been shown to be a good inducer of Thy-1 antigen expression (54). Its presence in a BSA gradient could artifically raise the background level of Thy-1 positive cells abrogating any chance of monitoring low levels of conversion. BSA stock solutions used were screened for the presence of endotoxin by the highly sensitive Limulus lysate test (179). BSA solutions which were negative were employed to construct the BSA gradients for cell separation or as controls for TF and butyrate induction.

Biological reagents such as antiserum and thymic factors may influence the consistency of results obtained with the *in vitro* conversion assay. Though prepared similarly, different lots of antiserum will have

different titers and affinities. Therefore, they may behave quite differently from one assay system to another as well as behaving differently within one assay system (Letarte--personal communication, 186,187). In the various lots of antiserum tested here, all of which have been prepared by the same method, few differences have been observed (results not shown).

Finally, an even greater threat of variation exists in the use of thymic hormones. The wide variety of isolation procedures currently in use lead to the purification of factors of various sizes and properties (71,74,76). Crude fractions also harbor a large spectrum of contaminants which may affect the results of the assay.

Many substances induce the expression of Thy-1 antigen on precursor T cells in addition to thymic factors. These include cAMP, dibutyrl-cAMP (DB-cAMP), Poly A:U, endotoxin (54), prostaglandin E_2 (57), ubiquitin (65), neuraminidase (63), and thymus RNA (188). The common denominator which most of these agents appear to possess is the ability to raise intracellular levels of cAMP. DB-cAMP was shown to be effective at lower levels than cAMP itself presumably due to the cell's increased permeability to the organic acid linked cyclic nucleotide (54). However, Wright (83), in an examination of the morphological and growth rate changes induced by DB-cAMP in Chinese hamster ovary cells, found that his control, sodium butyrate alone, was also effective in eliciting change. Subsequently, others have shown that sodium butyrate produces reversible changes in morphology, growth rate, and enzyme activities of several mammalian cell types in culture (90). Some changes mimic the action of DB-cAMP, while others are unique to the fatty acid. Since the induction of Thy-1 expression it thought in some manner to be mediated

by cAMP, sodium butyrate was employed as a putative inducing agent in the *in vitro* conversion assay. The results described here indicate that butyrate at low concentrations does mimic the action of cAMP and TF. Butyrate treated bone marrow and spleen cells of the low density fractions of BSA gradients express Thy-1 antigen in approximately the same percentage of cells as those induced by TF treatment (Table IV). In addition, anti-Thy-1 sera can be absorbed with butyrate treated marrow cells to the same degree as with TF treated marrow cells (Figure 1).

Other correlations can be made between butyrate induction of Thy-1 and butyrate elicited changes in certain mammalian cell culture systems. These include induction of differentiation in another hemopoietic system, induced morphological alterations, changes in ganglioside levels, and inhibition of induction by certain transcriptional and translational inhibitors.

It has been shown that butyric acid is a potent inducer of erythropoiesis in cultures erythroleukemic cells (102). Erythroid differentiation, as measured by the percentage of cells containing hemoglobin before and after treatment, was induced by 1 mM butyrate, a concentration which is comparable to the one used to induce Thy-1 expression in the experiments reported here. These investigators have postulated that butyrate may act directly on the cell membrane in light of its lipophilic nature. Others have actually demonstrated morphological alterations of the cell membrane in HeLa cell cultures upon treatment with millimolar concentrations of butyrate (93,97). Normally round, or polygonal HeLa cells extended long neurite-like processes and assumed a more fibroblastoid shape when incubated in the presence of 2.5-5.0 mM butyrate. Similarly, it has been postulated that TF induced Thy-1

expression may be the result of a membrane rearrangement (63).

Ganglioside levels have also been shown to change in response to butyrate treatment (92,96). In HeLa cells, the levels of the glycosphingolipid G_{M3} increase three to five fold, while the levels of the other major gangliosides remain constant (92). This was due to the induction of a specific sialyltransferase, the activity of which was raised twenty-fold (94). It has been proposed that the antigenic determinant of Thy-1 is carried by a ganglioside (27). It is possible, therefore, that an alternate mechanism of butyrate (and TF) induction is to raise the levels of a particular ganglioside, in this case Thy-1 glycolipid or one of its precursors, and that this increase leads to the expression of Thy-1 on the cell's surface. An attempt has been made to document a similar ganglioside increase in the *in vitro* conversion assay using radioactively labeled galactose and glucosamine to monitor newly synthesized ganglioside (results not shown). However, equivocal results have been obtained primarily due to the small number of cells available and to the small percentage of stem cells found even in enriched fractions from BSA gradients or nylon wool columns. A more homogeneous population of cells will be needed before changes in ganglioside levels can be detected.

Further evidence that TF and butyrate may act in a similar fashion to induce Thy-1 is the fact that both TF and butyrate induction of differentiation have been found to be sensitive to treatment with inhibitors of transcription and translation. Actinomycin D and cycloheximide were shown to inhibit the appearance of the alloantigens TL and Thy-1 which were normally expressed upon TF treatment (64). Butyrate induced morphological alterations and increased in ganglioside levels were

inhibited by similar levels of these two inhibitors in HeLa cell cultures (93,189).

In most systems where butyrate has been shown to induce some type of differentiation, the effect has been found to be quite specific for short, unmodified, straight chain fatty acids. HeLa cell modifications occurred only with C3, C4, or C5 saturated fatty acids (30,93), and erythroid differentiation was found almost exclusively with butyrate (102). Further specificity was demonstrated by the inability of isobutyrate to elicit characteristic changes (83,100,102). Possible additional fatty acid inducers in the *in vitro* conversion system have not been extensively examined. Acetate (2 mM) was utilized as a control in some experiments and was found to occasionally induce low levels of Thy-1 expression. Further experimentation is necessary to clarify these results.

The molecular mode of action of butyrate or TF is unknown. Currently, it is thought that TF induction is mediated by the second messenger cAMP. This is also thought to be true of some, but not of all, cases of butyrate induction. In addition, butyrate treatment has been shown to cause a decrease in DNA synthesis and a rapid acetylation of specific histones in both HeLa (100) and erythroleukemic (104) cell lines. It has also been postulated that prolonged butyrate treatment may synchronize cell cultures in the S (100) or G1 (93) phase of the cell cycle. Which, if any, of these documented effects of butyrate treatment induces the expression of Thy-1 on a precursor cell remains to be determined.

While butyrate is not the physiological inducer of Thy-1 expression on precursor cells migrating to the thymus, there are advantages

to studying this system with butyrate rather than TF as an inducing agent. It is chemically defined and easily obtainable in large quantities. Though the chemical definition of various thymic factors is now known (71,74,76), their availibility is still limited. Furthermore, butyrate has been shown to exert its effects on defined systems such as on differentiation of erythroleukemic cell lines. It may be possible to draw preliminary parallels between such systems, where cell quantities and homogeneity do not pose additional problems, and Thy-1 induction.

A third area to consider when examining the reproducibility of the Thy-1 induction assay is the target cell of the inducing agent, the precursor T cell. Identification of this cell in bone marrow or spleen has not been made due to inadequate separation of the cell from highly heterogeneous populations and its presence in such low quantities. To obtain a larger, more homogeneous population, one of two approaches can be taken. The first would be to obtain a cleaner separation of precursor T cells than is possible with currently used procedures. A second approach would be to artificially raise the number of prothymocytes present prior to isolation.

It has been attempted here to improve precursor T cell separation with the use of a parameter other than density to fractionate the cell population. Adherence to glass and nylon wool is a functional rather than a physical property which has been found effective in the enrichment of both B and T lymphocytes from spleen (160). The results reported here show that adherence to columns can also be useful in the separation of prothymocytes from bone marrow and spleen (Tables V and VI). Precursor T cells are found in the nylon wool nonadherent fraction as

are mature T cells. Others have documented similar findings (133). Still this procedure yielded a population of approximately 20% Thy-1 inducible cells, the same percentage obtained with density centrifugation. Preliminary experiments with bone marrow suspensions, however, have shown that, if two separation techniques are combined, with density centrifugation followed by incubation of the 21% and 23% layers on nylon wool, approximately twice the concentration of precursors are found in the nylon wool eluted fraction (results not shown). It appears, therefore, that separation on the basis of two distinct parameters may increase the percentage of precursor cells found in a particular population. Investigators in myelopoiesis have successfully explored such an approach using two physical parameters (density and volume) for separation (69,156).

It has been proposed that stem cells are viable replicating cells which exist in transitional states of various sizes (115). This would make separation by any one physical parameter very difficult. Moore et al. (130), using fetal liver and adult bone marrow cells separated by BSA gradients, obtained an even distribution of *in vivo* colony forming units in each of five different fractions. This finding suggests that the pluripotential stem cell may be found in various transitional states. Preliminary evidence suggests that this may also hold true for precursor T cells. When the normally noninducible cells of the 25% layer of a BSA gradient were further separated on a nylon wool column, it was found that ten percent of the cells in the nylon wool eluted fractions were induced to express Thy-1 (results not shown). Perhaps it will be necessary to find another more specific parameter in order to separate all progenitor T cells from a given population.

The number of stem cells committed to a certain line of hemopoiesis is manipulable by artificial means. For example, a simulated altitude increase produced hypoxia in mice which subsequently increased the demand for erythrocytes and the level of erythropoietin sensitive cells (190). An analogous situation exists in lymphopoiesis which may be useful in raising the numbers of precursor T cells in bone marrow or spleen prior to their isolation. It has been found that bone marrow from AKR mice with spontaneous thymomas have a 10-15 fold increase in the number of cells which equilibrate with the low density (10-23%) fraction of BSA density gradients (143). These cells have been shown to contain terminal deoxynucleotidyl transferase (TdT), an enzyme which has been found mainly in the thymus and bone marrow (141) and which has been proposed as a marker for precursor T cells (139,143). Treatment with thymopoietin made this increased population of TdT positive cells sensitive to anti-Thy-1 sera and complement (139), suggesting that they are prothymocytes. It appears that leukemic mice may be a source of larger quantities of precursor T cells than normal mice and that they might be useful for large scale prothymocyte isolation.

Most biological assay systems are subject to a certain degree of variation due to the large number of known and unknown parameters involved. The Thy-1 conversion assay is no exception. However, it has been shown here that this assay can be reproducible if a more sensitive method is used to quantitate the numbers of cells included to express Thy-1 than has been employed previously. If a more homogeneous population of precursor cells can be obtained either by manipulation or by improved separation and a chemically defined inducing agent such as butyrate employed, the Thy-1 conversion assay may be quite useful in elucidating some of the steps involved in cellular differentiation.

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