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Development of a model system to study the role of thymic nurse cells in T cell development

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DEVELOPMENT OF A MODEL SYSTEM TO STUDY THE ROLE OF THYMIC NURSE CELLS IN T CELL DEVELOPMENT

by

Mark T. Pezzano

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1992

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract
THE DEVELOPMENT OF A MODEL SYSTEM TO STUDY THE ROLE OF THYMIC NURSE CELLS IN T CELL DEVELOPMENT
by
Mark Pezzano

Advisor: Associate Professor Jerry Guyden

Thymic nurse cells (TNC) are large thymic epithelial cells which contain 20-200 developing thymocytes within specialized vacuoles in their cytoplasm. The purpose of the uptake of thymocytes by TNCs is unknown. However, TNCs have the capacity to present self-antigens, which implies that they may serve a function in the process of thymic education. We report here the development of two cell lines, which together constitute a model system with which to study the role of TNCs in T cell development. The first cell line, called TBL-1, expresses some characteristics of early T cells. TBL-1 occurred spontaneously from mouse thymus organ cultures derived from C57BL/6 mice. After cloning, the only T cell surface marker expressed by TBL-1 is Lyt-1. Analysis of the T cell antigen receptor (TCR) genes shows that they rearrange the α and γ genes and express full length α and γ mRNA transcripts, thus the δ gene is completely deleted. An immature 1.0 Kb β mRNA is transcribed from an unrearranged β gene. TBLs cannot produce either an α/β or γ/δ TCR. TBLs thus have little potential to mature along any known T cell developmental pathway. They do, however, specifically bind to both freshly isolated thymic nurse cells and the second cell line in our model system (MP5). The SVT-MP5 cell line is an SV40 transformed thymic nurse cell line derived from C57BL/6 mice. This cell line has been characterized using a panel of antibodies against markers used to identify TNCs. MP5 was shown to express A2B5, class I and class II MHC, and cytokeratin. In addition they were shown to bind and internalize both fresh thymocytes and the TBL-1 cell line. Internalization of thymocytes is unique to SV40 infected TNCs and TNCs within the thymus. The MP5 and TBL-1 cell lines together represent a model system with which we can determine the proteins involved in the internalization process as well as the role of TNC internalization in T cell development.

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There are many people who have been important to the successful completion of this thesis, but none that I would like to thank more than my parents Evelyn and Arnold Pezzano. The relationship that you have with your parents goes through an evolution of sorts. When you are a small child they are the focus of your life. As time goes on you develop other relationships, and friends become a significant part as well. When you reach puberty there are times when you think that there is just no way that your parents could understand what you are experiencing. It is only later on that you realize the wisdom of their life experience, and the depth of their commitment to and love for you. Words can not do justice to the feelings of appreciation and love that I have for my parents. It is their constant encouragement, sacrifice, and unconditional love that has made me the person that I am, allowing me to pursue and ultimately complete this degree. Thank you so much Mom and Dad, I hope that I can some day be as wonderful a parent to my children as you have been to me.

I would also like to thank my mentor and friend Jerry Guyden. You have taught me many things, Jerry, more than either of us realize. You have not only taught me about science, but also, and maybe more significantly about people. I hope that I can continue your work in shaping the minds of young and old people and making them realize how important it is to respect and admire the diversity in this world.

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INTRODUCTION

Background

The two major functional cell types of the immune system, B and T lymphocytes, are both derived from cells originally found in the bone marrow or fetal liver. Pre-T and -B cells then migrate to the thymus and spleen, respectively, where they differentiate into functional cells of the immune system. Mature T cells are divided into two subsets on the basis of their function in the immune response. These subsets include cells capable of cytotoxic ($CD8^+$) and helper ($CD4^+$) activity. A third subset with the ability to suppress or control the immune response may also exist, but to date no suppressor cell clones have been isolated. On the contrary, most cellular immunologists consider it likely that a subpopulation of the $CD8^+$ lymphocytes is responsible for suppressor function. The existence of more than one functional cell type increases the difficulty of understanding the sequence of differentiation steps as they occur in the thymus. The development of the fluorescence activated cell sorter (FACS) and monoclonal antibodies to T cell specific antigens, as well as the discovery of the T cell antigen receptor (TCR), has allowed the characterization of many different subsets of T cells within the thymus, and has produced much information about the processes involved in T cell maturation. In the currently accepted model for T cell ontogeny, the T cell precursors migrate from the bone marrow and colonize the thymus on day 11 in the fetal mouse. These very early T cells express only the Thy-1 and Lyt-1 cell surface markers and are termed "double negative" cells (see appendix 1). They are termed double negatives because they express neither CD4 nor CD8. These double negative cells are incapable of

recognizing or responding to antigen because they do not express either TCR or the associated signal transducing complex CD3. Double negative cells then turn on and express both CD4 and CD8, thus becoming "double positive" thymocytes. Several groups have shown that a $CD4^+$ subset, which has not rearranged any of the genes for the T cell antigen receptor, may be an intermediate between the double negative cells that first colonize the thymus and the double negative cells which have rearranged the TCR genes (1). On the other hand evidence exists for a $CD8^+$ $CD4^-$ intermediate between the double negative and double positive stages. This subpopulation was distinguished from mature single positives because it had a lower level of surface TCR expression than the mature cells (2). At some point just before or during the double positive stage of development, thymocytes begin to express the α/β type TCR and the associated CD3 complex. It is also believed that restriction occurs during the double positive stage. Restriction is the process by which T cells learn to distinguish self from non-self. Thymocytes then lose the expression of either CD4 or CD8, and become mature single positive cytotoxic or helper T cells, respectively.

The mature repertoire of foreign antigen-specific, self MHC-restricted T cells is selected in the thymus. The TCR genomes of all individuals can code for receptors that can recognize virtually any antigen (self or foreign) in association with any MHC molecule (also self of foreign). Two sets of genes exist, $\alpha\beta$ and $\gamma\delta$, encoding two different receptors expressed on different populations of T cells. The mature $\alpha\beta$ expressing cells are the classic peripheral T cells, consisting of the cytotoxic and helper lineages, while the $\gamma\delta$ cells have a more limited repertoire of TCR types. The $\gamma\delta$ T cells are thought to function in immunosurveilance of epithelial tissue

such as the skin and the lining of the gut. The incredible diversity of TCR types is generated by a unique recombination process controlled by the two recombinase genes RAG-1 and RAG-2 (3,4). Each TCR gene is composed of multiple genetic elements, V,(D), J, and C, which rearrange to assemble the somatically functional gene. Diversity is generated from the large number of different members of each family of elements, as well as from mechanisms that alter the DNA at the joining regions of the V-D, D-J and V-J elements. Successful rearrangement of the γδ type receptor excludes the possibility of producing an αβ TCR and vice versa because the entire δ gene is actually located between the variable and joining regions of the α gene. The joining of the V and J regions deletes the δ gene. After different receptors are expressed on the surface of different clones of developing αβ T cells, the repertoire is modified or shaped by two related selection processes. The selection process, by which the T cell repertoire becomes self-MHC restricted, is called restriction or positive selection. Negative selection then eliminates or inactivates potentially autoreactive clones, ensuring that the mature T cells are self-tolerant. These selection processes are due to the selective growth or death of individual cells and occur within the entire population of developing T cells.

It follows that the microenvironment of the thymus is required for both inducing precursor cells to differentiate into functional T lymphocytes, and for selecting among the developing cells those with the ability to recognize antigen in the context of self major histocompatability complex. The actual sequence of steps, starting from the precursor cell as it enters the thymus and ending with the release of a functional T lymphocyte into the periphery remains obscure. However, the diversity of the subpopulations found within the thymus suggests that it is a very

complex process. The engagement of MHC during positive selection and during antigen presentation in the periphery is mediated by the TCR associated with CD4 or CD8. The variable regions of the TCR are believed to contact the processed self or non-self antigen and the associated polymorphic regions of the MHC complex while CD4 and CD8 contact the nonpolymorphic regions of MHC class II and class I, respectively (5). The receptors on the $CD4^+$ cells generally recognize peptides derived from endocytosed antigens, while $CD8^+$ cells generally recognize peptides derived from cytoplasmic antigens such as viral proteins (6). Activated $CD4^+$ cells release lymphokines that result in the stimulation of B cells to produce antibodies (TH2) or the activation of macrophages to mediate delayed-type hypersensitivity (TH1). Activated $CD8^+$ cells are stimulated to release large quantities of γ-interferon and become cytotoxic for cells expressing foreign antigen (7).

The development of self tolerance in both $CD4^+$ and $CD8^+$ cells involves the clonal deletion of self reactive T cells and thus is termed negative selection. The idea of clonal deletion was first suggested by experiments using limiting dilution analysis to show that the frequency of T cells able to react to a specific antigen is greatly reduced in tolerant animals (8). Later, another group showed that mice that did not express a particular MHC class II molecule (I-E), expressed a particular variable (V) segment of the β chain TCR (Vβ17a) on 9-14 % of their peripheral T cells, making them a major subpopulation. However, this subpopulation is greatly reduced (<1%) in F1 crosses with mice that do express I-E. They also demonstrated that within the thymus, Vβ17a cells were present in normal numbers at the double positive stage while being greatly reduced in the single positive mature subsets (9). These results suggest that the Vβ17a

expressing T cell clones are deleted somewhere between the double positive and single positive stages. Since both the CD4 and CD8 single positive Vβ17a expressing types were reduced, and only CD4 interacts with MHC class II (I-E) molecules the deletion must occur at the double positive stage. Kyewski et al. presented more evidence for this conclusion when they showed that it is the double positive subpopulation which is found rosetted around the thymic stromal cells which are thought to be involved in negative selection (10). It should also be noted that injecting large amounts of CD4 monoclonal antibodies into mice recovering from irradiation and bone marrow reconstitution prevented the development of $CD4^+8^-$ cells while allowing the appearance of $CD4^-8^+$ subpopulations expressing the Vβ17a TCR (11). These results demonstrate the necessity of CD4 for the deletion of $CD8^+$, $V\beta17a^+$ cells and further support the notion that clonal deletion of Vβ17a expressing cells requires both the αβ TCR and CD4 at the double positive stage of T cell ontogeny. It was also shown that MHC II is necessary for the deletion of $V\beta17a^+$ clones at the double positive stage (12).

A second system, independent of the Vβ17a model, has also been employed in an attempt to elucidate the mechanism of tolerance induction. This system relies on the use of TCR transgenic mice, and the expression of the male specific antigen H-Y (13). Under normal conditions female mice produce a cytotoxic T cell response when injected with male H-Y expressing spleen cells. Clones of the H-Y, MHC I (D^b) specific $CD8^+$ T cells have been produced. In addition the genes for the H-Y specific α β TCR have been cloned. Transgenic mice were produced which expressed very high levels of the H-Y specific αβ TCR on their T cells. When peripheral T cells were tested for their ability to respond to H-Y plus D^b,

14% of the $CD8^+$ cells were stimulated to proliferate in females, while almost none (.004 %) were stimulated in males (13). When thymic T cell populations were examined in the transgenic mice, males had 10 fold fewer cells than females which was attributed primarily to diminished numbers of double positive cells. The double negative cells were present in normal amounts and expressed high levels of the αβ transgenic receptor. These results suggest that large numbers of double positive cells are deleted in male mice in response to the presentation of self H-Y antigen in the context of D^b, resulting in the diminished number of peripheral cells which can be induced to proliferate.

Separate from the negative selection process described above, a second process is involved in shaping the mature T cell repertoire. In this selective step, those T cells which are able to recognize the particular allelic forms of MHC molecules expressed in the thymus are selected to develop further. This event is known as positive selection. This process was first suggested by the results in studies of radiation-induced bone marrow chimeras. In these studies mice of one type of MHC are irradiated to destroy all of the lymphoid cells, including bone marrow derived dendritic cells and macrophages. The radiation does not effect the stromal epithelial cells of the thymus. The mature T cells produced can only recognize antigen in the context of the MHC allele expressed on the epithelial cells in the irradiated thymus, even though the bone marrow used as a donor to reconstitute the lymphiod cells is from a mouse expressing a different MHC allele (15). Further evidence for the existence of a positive selection mechanism came again from the use of antibody blocking experiments, and TCR transgenic mice. In these experiments (MHC A X MHC B) F1 mice were treated during development with high

doses of antibody against MHC type A to mask the antigenic epitopes. The resulting T cells were much better able to recognize antigens in the context of type B MHC molecules than type A. This suggested that recognition of the particular allelic form of the MHC molecule was required for the development of T cells able to recognize specific foreign antigens bound to that MHC molecule (16). Similar studies were performed using CD4 and CD8 monoclonal antibodies and the result was the selective absence of either the $CD4^+$ or $CD8^+$ mature subpopulations, respectively (11). This implies that CD4 and CD8 are also necessary for positive selection to occur. One exciting piece of evidence which seems to suggest that tolerance induction and positive selection are, in fact, unique events, is that in antibody blocking experiments animals are completely tolerant of a particular MHC allele even when blocking with antibodies has prevented positive selection (17).

Experimental evidence from the use of αβ TCR transgenic mice shows that the selection for expression of TCRs that recognize self MHC alleles also occurs at the double positive stage (18, 19). Together this evidence suggests that positive selection results in the splitting of the T cell population into its two major functional classes $CD4^+$ (T helper) and $CD8^+$ (T Killer/Suppressor) as a result of recognition of MHC class II and MHC class I molecules, respectively. These MHC molecules are expressed on the surface of radiation insensitive stromal epithelial cells, and are recognized by αβ TCR expressing double positive cells. Any particular receptor can end up on either a $CD4^+$ or $CD8^+$ mature cell depending on its ability to interact with a particular MHC molecule.

Little is known, however, about the role of the interactions between lymphoid cells and particular types of nonlymphoid cells in the

microenvironment of the thymus. The development of new techniques to isolate specific, *in vivo* pre-existing, complexes between thymocytes and several stromal cell types of the thymus affords a new approach to the investigation of the intrathymic events of T cell differentiation. (20,21) These multicellular complexes include thymocyte rosettes, which are thymocytes interacting with I-A negative macrophages (Mϕ) or I-A positive dendritic cells and thymic nurse cells (TNC) which are I-A positive H2 positive thymic epithelial cells (21,22,23).

There is a significant body of evidence that suggests the interactions between thymocytes and cells of the stroma may be necessary for T cell education. The details of this process are not known, but it is believed that stromal cell presentation of self antigens to thymocytes plays an important role in the selection process (24, 25). The three distinct populations of antigen presenting cells in the thymus include macrophages, dendritic cells and thymic nurse cells (26). Macrophages and dendritic cells have been shown to be fully capable of stimulating the release of lymphokines from a self hemoglobin specific T cell clone as a function of antigen presentation. This shows that the Mϕ/dendritic cell lineage can present processed self antigens, a necessary step for tolerance induction. TNCs, however, require the addition of IL-1β to facilitate the presentation of antigen (27). It is thought that the binding of self antigens in the context of major histocompatability complex Ia to the TCR initiates the proliferation of target T cells. The binding specificity is determined by the specific TCR expressed and results in either clonal activation or deletion depending on how well the TCR active site binds the self/Ia complex (28).

Experiments on bone marrow reconstituted radiation chimeras show a sequential appearance of thymocyte interactions with macrophages

followed by interactions with TNC and dendritic cells. This suggests an essential role for these complexes during the early stages of intrathymic T cell differentiation (20). It is thought that the cortical thymic epithelial cells are responsible for positively selecting those thymocytes which have a strong affinity for self MHC molecules, possibly by making them insensitive to corticosteroids, which may induce apoptosis. The double positive lymphocytes are then thought to migrate to the cortical/medullary junction where they interact with bone marrow derived Mϕ/dendritic cells. It is at this point in ontogeny that negative selection occurs. Those lymphocytes which have a very high affinity for self MHC or MHC plus processed self antigens are deleted from the population. Evidence for this model of self MHC restriction and tolerance induction comes from several sets of experiments involving reconstitution studies and transgenic animals. When deoxyguanosine treated fetal thymuses (deoxyguanosine destroys the native T cells and bone marrow derived Mϕ/dendritic cells) of strain A mice are injected into athymic (nude) mice of strain B the resulting T cells are restricted to type A MHC, but are not tolerant to the MHC molecules of strain A. When deoxyguanosine is not used, then tolerance to A is attained (29). Since the epithelial cells of the thymic stroma are not affected by deoxyguanosine treatment, these results suggest that it is the bone marrow derived Mϕ/dendritic cell lineage which is responsible for tolerance induction while the epithelial cells are involved in positive selection. In another experiment, transgenic mice were produced which only expressed MHC class II (I-E) in its cortical epithelial cells but not in the cells of bone marrow origin. When peripheral T cells of these mice were examined for their expression of Vβ17a$^+$ TCRs (Vβ17a recognizes unknown self

antigens in the context of I-E), they were shown to exist, and to retain their auto reactivity to B cells expressing I-E (30). These results suggest that negative selection occurs as the result of interactions between Mϕ/dendritic cells but not stromal epithelial cells. Thymic epithelial cells have also been shown to produce thymic hormones, which may promote T cell maturation (33). These hormones have been well characterized biochemically but their physiological roles are unknown. They have been given various names, including thymosin, thymopoietin, thymulin, and thymic humoral factor. These thymic hormones have been shown to promote the appearance of some lineage specific surface molecules on bone marrow cells or immature T cells *in vitro*. They were also shown to enhance T cell functional responses, such as proliferative responses to lectins. Thymic stromal cells may also secrete interleukin-7 (IL-7), a cytokine that may stimulate the proliferation and maturation of developing T cells in the thymus. It should be noted however, that no thymic hormone or cytokine has been shown to support the development of TCR expressing mature T cells from bone marrow cells or immature cortical thymocytes *in vitro*.

Focus of Thesis Work

Thymic nurse cells are unique stromal epithelial cells found in the cortex of the thymus. They have been identified in a variety of organisms including humans (31), mice (21), rats, sheep and chickens (32). From 20 to 200 proliferating lymphocytes may be found within specialized vacuoles in the TNC cytoplasm (22). Very little is known about how these thymocytes come to reside in TNCs, or what changes occur in the cells as a result of this unique type of interaction. Thymic nurse cells were first discovered by Wekerle and Ketelson (1980). These initial studies presented evidence of

the epithelial nature of these cells. Their work also showed that trapped lymphocytes were completely isolated from the thymic microenvironment by the TNC plasma membrane. TNCs express both class I and class II MHC antigens on their cell surface, as well as on the vacuolar membrane surrounding the internalized T cells (21, 33). They do not express lymphocyte specific markers such as Thy-1, Ly-1 (CD5), CD4 or CD8. Attempts at the characterization of TNC thymocytes have shown them to be a very heterogeneous population (33), and most of the cells have been found to be nonfunctional (34). It should be noted, however, that it is not possible to isolate a totally pure population of TNCs with internalized lymphocytes. There are always contaminating lymphocytes in the isolation procedure. Both immature and mature T cell phenotypes have been identified within TNCs. A low level of cytolytic activity was obtained in some TNC lymphocyte preparations (34) while others show a small population of cortisone-resistant cells expressing the phenotype of mature precursors of the helper T cell lineage (33). This finding may suggest that some degree of differentiation is occuring within the microenvironment of the thymic nurse cell, where immature non-functional cells are being stimulated to differentiate into mature functional cells.

Although TNCs have been shown to be able to present antigen to T cells, no information is available describing the interactions between the TNCs and the lymphocytes found within their cytoplasm. The current method for isolating TNCs from fresh tissue relies on their very large size and increased density due to internalized lymphocytes. 70-80% pure populations of TNCs can be isolated by repeated enrichment on fetal calf serum density gradients (22). Unfortunately, the total number of cells

recovered per mouse thymus is only about 10^5 cells. This low yield, combined with the fact that there are always contaminating lymphocytes, makes it very impractical to work with fresh cells. In addition, the cells only survive for a short time in culture.

Very little is known about the early stages of thymocyte development. In addition, not much information about the role that interactions between non-lymphoid cells of the thymus and developing T cells play, has been reported. In an attempt to clarify our understanding of these early events in T cell development, we have isolated T cell lines (TBLs) from very young (13-14 day old) fetal thymi of C57BL/6 mice. Colonies of cells grew spontaneously from dissociated thymi and the resulting cells were analyzed for cell surface Thy-1 expression. $Thy1^+$ cells were cloned by sorting one cell per well in 96 well plates using a FACS. The cells were grown to confluency and analyzed again for a panel of cell surface markers. FACS analysis showed the cells to be of an Lyt 1^+, double negative ($CD4^-$ $CD8^-$) phenotype that had lost Thy1 expression in culture. This phenotype suggests that they were derived from an immature T cell subpopulation. TBLs have been stable in culture for more than four years. Northern blots of whole cell RNA screened with probes for genes of the T cell antigen receptor (TCR) complex show a previously unreported pattern of gene expression. No surface TCR was expressed. Several groups have demonstrated that freshly isolated immature double negative T cells could be induced to differentiate into double positive or even single positive cells by placing them in organ culture (35), in contact with thymic epithelial cell lines (2), or by injecting them, intrathymicly, into congenic host mice (36, 37). It was originally thought that it would be possible to develop a complete *in vitro* system to study T cell development. It was

thought that immature T cell lines could be produced that, if placed in the proper environment, could differentiate into double positive cells, rearrange and express the TCR and ultimately become functional single positive T cells. That was the idea behind developing TBLs. Unfortunately, we now know that this will never be possible. Cell lines have been developed by Alt et al. which express the recombinase genes (RAG-1 and RAG-2), but it has been shown that these genes are selected against in cell lines that express them, because they induce fatal mutations if they remain active constitutively. In the thymus, developing thymocytes only turn on the recombinase genes for a very short period of time, during which the TCR genes are rearranged (Alt, F. personal communication). The most exciting characteristic of TBLs is their interaction with thymic nurse cells. Co-culture of TBLs with freshly isolated thymic nurse cells results in the formation of cell clusters, with the TBLs completely covering the thymic nurse cells after 24 hrs, much like fresh lymphocytes.

This fact prompted us to attempt to develop a thymic nurse cell line with the potential to bind lymphocytes, thus providing a complete *in vitro* model system to study TNC function, as well as the mechanism of binding and internalization. As discussed above, it is very difficult to isolate large numbers of fresh TNCs without contaminating lymphocytes. Thus, it has been impossible to study the binding and internalization process or the role of TNCs in T cell development. Other groups have developed model systems to study lymphocyte/stromal cell interactions, but none has developed a TNC line with the ability to internalize lymphocytes in culture. Tatsumi et al. have developed a thymic stromal cell line called MRL 104.8a which has been shown to induce differentiation of double

negative thymocytes into double positive, and even single positive thymocytes *in vitro* (38). Another study described the isolation of a stromal cell line, cloned from a spontaneous thymic tumor, that had the potential to bind freshly isolated thymocytes in culture (39). Hugo and Potworowski have developed a medulla derived epithelial cell line (E-5) which has the ability to form complexes with double positive thymocytes *in vitro* and may represent a model system to study negative selection (40, 41). Finally, Ezaki et al. discovered that a particular strain of rats, called BUF/Mna, develop spontaneous benign thymomas of epithelial origin at approximately eight months of age. Remarkably, these thymomas seem to be simply greatly enlarged thymi, which have 100 times more TNCs than normal thymi at that age. Also, the number of TNCs continues to increase, unlike normal thymi which have reductions in the number of TNCs after 2-3 months (42). These animals should allow the isolation of large enough numbers of fresh cells for study. Unfortunately, the same limitations to the system will exist, in that there will still be contaminating lymphocytes, and the TNCs die a short time after they are removed from the thymic microenvironment.

In an attempt to immortalize TNCs, in the current study, freshly isolated thymic nurse cells were infected with SV-40 virus. The cells which grew were cloned by limiting dilution, and one of the resulting clones termed SVT-MP5 was characterized using a panel of monoclonal antibodies which identified the cells as thymic nurse cells with SV-40 stably integrated into their genome. The lymphocyte binding potential of SVT-MP5 was then tested using a modified binding assay. MP5 was shown to specifically bind both the TBL-1 cell line and fresh thymocytes. In addition, MP5 was shown to internalize both fresh lymphocytes and the

TBL cell line in culture. This is the first report of the formation of TNC/lymphocyte complexes *in vitro*. This cell line along with the TBL cell line and freshly isolated lymphocytes represents a model system to study the binding and internalization process, as well as the role of TNCs in T cell development.

Methods

Mice. Breeding pairs of C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Three females were introduced into a cage with one male for 12 hours (overnight). The females were removed and examined 10 days later for pregnancy by abdominal palpation. Two weeks after birth, mice were dated and used in the following experiments.

Fetal Thymocyte Cloning. Cell suspensions were obtained from 13-14 day old fetal thymus lobes of C57BL/6 mice by mechanical dispersion in growth media. The cells were washed once with Gey's Complete Salts solution to remove erythrocytes. The remaining cells were suspended in growth media and seeded at 5×10^5 cells per ml in 96-well flat bottom dishes (Becton Dickinson, Oxnard, CA). The cultures were fed every other day with growth media. Massive cell death was observed on the third or fourth day of culture but spontaneous growth was detected 10-12 days later. Cells were removed from wells containing colonies, resuspended in 6 well plates and grown to confluency. Long term cultures were seeded at 1×10^6 cells per ml and maintained in T-75 flasks (Corning, Corning, NY). The resulting cells were stained with anti-Thy-1 antibody, and Thy-1^+ cells were sorted at one cell per well using a FACS (Becton Dickinson, Sunnyvale, CA). Cell lines obtained using this method were considered to be unique when isolated from different thymic lobes.

Antibodies and Immunofluorescence Staining. (For thymocytes) Fluoresceinated monoclonal antibodies against Thy-1, Lyt-1 (CD5) and Lyt-2 (CD8) were obtained from Becton Dickinson (Mt. View, CA). The hybridoma producing rat monoclonal antibody against L3T4 (CD4) (Clone GK1.5) was purchased from American Type Culture Collection (Rockville, MD). Anti-mouse CD3ε and CD45 were purchased from Boehringer Mannheim. (For thymic nurse cells) TNCs were stained with anti-A2B5 (American Type Culture Collection, A2B5 clone 105), a rabbit polyclonal anti-keratin antibody (Dr. T.-T. Sun, New York Medical Center), anti-Ia^p (crossreactivity to b) and anti-$H2^b$ (Accurate Chemical). A hamster polyclonal anti-T antigen antibody was used to stain TNCs as an assay for transformation (provided by Dr. Robert Carrol, New York Medical Center). All antibody preparations were airfuged prior to use, to remove aggregates. Affinity-purified second step reagents, mouse anti-rat immunoglobulin and goat anti-mouse immunoglobulin, were obtained from Bionetics Lab (Charleston, SC) as fluorescein conjugates. Fluoresceinated rabbit anti-hamster antibody was purchased from Cappel laboratory (Malvern, PA).

Immunofluorescence staining was performed as follows: Single cell suspensions were adjusted to 1 X 10^6 cells/ml in cold 5 mM HEPES-buffered Hank's balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA) (Sigma) and 1% sodium azide (NaN), pH 7.4. One ml of the cell suspension was incubated for 15 minutes at 4^oC with a saturating concentration of monoclonal antibody. After three washes, the cells were incubated with the appropriate second step antibody for 15 minutes, washed, and fixed in 1% buffered paraformaldehyde. As a control, cells were incubated with HBSS instead of the first step antibody prior to staining with the second step reagent. For cytoplasmic staining,

the cells were fixed with a 1:1 methanol/acetone mixture for 3 minutes before performing the staining procedure. Treated cells were viewed under phase and fluorescence optics on a Zeiss fluorescence microscope equipped with epi-illumination.

Northern Blot Analysis. Total cellular RNA was isolated from cells in 4M guanidine thiocyanate. 10 μg of RNA was separated overnight at 560 volt hours on a 1.0 % agarose gel containing formaldehyde in 0.2 M morpholinopropanesulfonic acid-acetate before blotting onto nitrocellulose. The blot was hybridized to a ^{32}P-labelled probe made with Cα (a gift from Dr. J. P. Allison), or Cβ, isolated from the MSDβ plasmid (a gift from Dr. Tom St. John, Standford University), or Cγ, isolated from a cDNA library cloned into pUC8 by Dr. David Asarnow in the laboratory of Dr. J. P. Allison., or Cδ, isolated from a λgt10 clone of δ in pUC18 (a gift from Dr. M. M. Davis).

Southern Blot Analysis. 10 μg of DNA was digested for two hours and separated overnight on a 0.9% TAE-agarose gel before blotting onto nitrocellulose. The blot was hybridized to a ^{32}P-labelled probe made with the same fragments used in the northern blot procedure described above.

Fresh Thymocytes. Cell suspensions were obtained from the thymus of two weeks to one month old C57BL/6 mice by mechanical dispersion in growth media. The cells were washed once with Gey's Complete Salts solution to remove erythrocytes. The remaining cells were suspended in growth media and seeded at 1 X 10^6 cells per dish containing a monolayer of TNCs.

Binding Assay. A modified version of the cell-to-cell adhesion assay (43) was performed. 10^4 TNCs were allowed to attach to the bottom of each well of a 24 well plate (Becton Dickinson). Equal numbers of ^{35}S-methionine-labelled (100 mCi per ml, 1129 Ci/mmol, New England Nuclear, Boston, MA) lymphocytes were added to each well and incubated overnight at 37°C. Unattached lymphocytes were removed by three washes with PBS, and the amount of radioactivity was determined by scintillation counting of that remaining in the wells. The percentage of bound cells was determined as a function of the total number of counts added to each dish.

Homing and Differentiation Experiments . Breeding pairs of congenic C57Bl/CyLy1a were obtained from the Jackson Laboratory (Bar Harbor, Maine). Three females were introduced into a cage with one male for 12 hours (overnight). The females were removed and examined 10 days later for pregnancy by abdominal palpation. Mice of various ages from 1 day to 1 month old were injected either intraperitonealy or intrathymically with 10^7 TBLs. TBLs originated from C57BL/6, a congenic strain at the Ly-1 locus. Thymic nurse cells isolated from these mice and their internalized lymphocytes were double stained with Ly 1.2 (which distinguishes TBLs from the host lymphocytes which are Ly1.1) and a panel of monoclonal antibodies against other T cell surface markers including CD4, CD8 and Thy 1.

Analysis of the growth potential of TBLS *in Vitro*. Whole RNA was isolated from TBLs using the procedure described in the section on

northern blots above. The resulting RNA was then run on an agarose gel containing formaldehyde, blotted to nitrocellulose and hybridized with nicktranslated probes produced from *c-myc*, *c-fos* and *c-myb*. The level of mRNA expression for these genes in TBLs was compared with known controls. For any of the oncogenes that showed an increased expression in TBLs, Southern blots were also performed, as described above, to detect rearrangements or amplifications of the DNA.

Thymic Nurse Cells. Thymic nurse cells were isolated using fetal calf serum density gradients (22). Briefly, thymi were minced and suspended in a 50 ml centrifuge tube containing phosphate-buffered saline (PBS). The PBS was removed and the large tissue fragments that settle at the bottom of the tube were subjected to two consecutive 5 minute digestions with 0.25% trypsin containing 1.5 mg/ml collagenase (Sigma, St. Louis, MO) at 37°C. The resulting cells were separated on a fetal calf serum gradient at 1 X g for 45 minutes. The gradient was repeated to increase the enrichment of TNCs. The cells were suspended in growth media containing RPMI-1640 (Gibco, Grand Island, NY), 2% Nu Serum (Collaborative Research, Boston, MA), 0.01 % 2-mercaptoethanol (Sigma), 1% each of gentamicin (Gibco) and L-glutamine (Grand Island Biologicals, Grand Island, NY), and 10% fetal calf serum (Sigma).

Infection of TNCs with SV40. TNCs were allowed to release enclosed thymocytes and to form monolayers before exposure to SV40 (strain Rh911, originally obtained from Dr. V. Defendi) at a multiplicity of 10 PFU/cell at approximately 5 X 10^4 cells/ml in RPMI plus 2 μg/ml polybrene. After an adsorption period of 30 minutes, the cells were

diluted with growth media and maintained in culture until the growth curve assay was performed.

Growth Curve (SVTNC). The growth curve was initiated approximately 14 days post infection. 1 X 104 cells were added to 25 mm round culture dishes and allowed to adhere to the dishes overnight. Three dishes were counted for each time point using a hemocytometer, and viability was determined by Trypan blue exclusion.

Results

Characterization of TBL-1

Isolation. The thymus was removed from 13-14 day old fetal mice and cells were dispersed into culture by mincing with two 18 gauge needles. Cells resulting from one thymic lobe were placed into one well of a 96 well plate containing 0.1 ml of growth media. The cells appeared to grow rapidly for 48 hr. at which time there was massive cell death. The cultures were fed every other day up to 14 days. Colonies of non-adherent lymphoblast " like" cells appeared between day 10 and day 14 of tissue culture. Originally, 19 lobes were isolated and placed into individual wells. Colonies were found in every well. The cells were grown to semi-confluency (dense but uncrowded) before being transfered to tissue culture flasks for bulk culture.

Since these cells grew in suspension and were thymus derived, it was reasonable to use anti-Thy-1 antibody for staining in an attempt to clone by flow cytometry. The results of this experiment are shown in figure 1A. Approximately 11% of the cells from the original isolates stained positive with the anti-Thy-1 antibody (live stained cells can be seen in panel 2). A single cell from this population was deposited into each well of a 96 well plate. Seventy five to eighty percent of the isolated cells resulted in viable clones. Five of the resulting clonal populations were recloned using limiting dilution. Ten of the clones obtained using this procedure were grown to confluency and frozen for future analysis. Five other clones were used in this study and have been maintained in culture for over nine months through serial passages. These cell lines represent

independent isolates because each was derived from a different thymic lobe. Further immunofluorescent studies (Fig. 1B) of one of these clones revealed that the cell surface expression of Thy-1 is lost by the time enough cells were recovered for reanalysis (1×10^6 cells were needed for each antibody staining, which takes approximately 3 weeks to obtain from a single cell). However, Lyt-1 was readily detected and was stably expressed after months of culture. These characteristics were similar to $B_{Ly\text{-}1}$ cells which grow spontaneously out of splenic lymphocytes. For this reason, we named these cells TBLs for (T)hymus derived (B)(L)y-1 cells. This idea was ruled out when the cells stained negative for IgM and Fc receptor, antigens usually associated with BLy-1 cells (44). They also show no staining with antibodies against IL-2 receptor, Ly-5, Mac-1, IgD and anti-124-40 a polyclonal anti- TCR antiserum (45) (Table 1). Together these data suggests that the cell surface phenotype, expressed by TBLs, is that of a very early T cell.

Growth Properties. All of the cell lines were isolated in media containing 10 % fetal calf serum. A test for media requirements (Fig. 2a) shows that growth is dependent on serum concentration. It should be noted that their growth rate remains relatively high at serum concentrations as low as 3 %. We thought this response might have resulted from the use of Nu serum, a serum supplement, in our growth medium. This possibility was investigated by testing the growth potential of TBLs in various concentrations of Nu serum (Fig. 2b). The growth rate of TBLs was shown to be unaffected by Nu serum concentration, as the cells grew equally well even when no Nu serum was present in the medium. The growth of TBLs with such low serum requirements suggests that they may be

neoplastic. The cells maintained their non-adherent rounded morphology (Fig. 3a) indefinitely in growth media. Clumps no larger than eight cells were found under normal conditions. Upon exposure to monolayers of thymic nurse cells (Fig. 3b,c), TBLs became attached within 24 hours. The TBLs completely cover the TNCs after 48 hours of exposure (Fig. 3d). This result is similar to that observed when fresh thymocytes are incubated with TNCs, but the phenomenon has never been observed with any other lymphocyte cell lines tested (Fig 11)

Rapid proliferation and long-term growth potential in tissue culture are properties of neoplastic transformation. The tumorigenicity of TBLs was tested by injecting 1 $x10^6$ cells intrathymically into a congenic strain of mice, B6-$Ly1^a$/ Cy (Lyt-1.1)(Table 2). A total of 18 mice were used in two independent studies. At the end of a 2 month period no visible signs of tumors were found (Tumor formation occurs within 7 days upon injection of neoplastic T cell lines). The vigor and behavior of injected animals appeared normal. All of the animals were sacrificed and thymuses were surgically removed for further analysis. The size of the injected thymuses were normal when compared to control animals. The thymic presence of transplanted TBLs was determined by immunofluorescence staining with antibody against Lyt-1.2. An average of 2-3 % of the total thymic cell population expressed the Lyt-1.2 antigen when isolated from injected animals. No staining was detected from uninjected controls. In addition, when the lymphocytes were analyzed in double staining experiments for the expression of Lyt-1.2 and CD4 or CD8, no $CD4^+$ or $CD8^+$ cells were recovered. This suggests that although TBLs persist in the thymus for several months after injection, they are not differentiating into more mature phenotypes. Similar experiments were

done by injecting the TBL-1 cells intraperitoneally, to test their migratory potential. Again these cells were found to be non-tumorigenic, and the TBL cells were shown to home to the thymus of congenic animals. Approximately 1% of the total thymic cell population was shown to express Lyt-1.2 (Table 2). These results suggest that TBLs are not tumorigenic *in vivo,* but may grow in culture as a result of the loss of some growth controlling element present in the thymus. The fact that TBLs home to the thymus is further evidence that they represent a very immature T cell lineage. Similar results have been documented with the B-Ly1 cell line. These cells have also been shown to nontumorigenic, after they home to the spleen when injected IP and their growth in culture seems to be the result of over expression of the *c-myc* gene (44).

***C-myc* Expression by TBL-1.** The fact that TBLs grow spontaneously in culture, yet are nontumorigenic when placed in the microenvironment of the thymus prompted further study of their uncontrolled growth *in vitro.* Due to the similarity between TBLs and B Ly-1 cells we decided to look at the expression and conformation of the *c-myc* gene which is amplified in B Ly-1 cell lines. Northern blot analysis revealed a 10 - 70 fold increase in *c-myc* mRNA expression (Fig. 4). Southern blot analysis showed that the reason for the increased expression was that the myc gene was amplified when compared with the actin control. Laser densitometry showed there to be 10- 70 more copies of the *c-myc* gene in different TBL clones when compared to controls (Fig. 5). This amplification and subsequent increased expression could account for the spontaneous growth of TBLs *in vitro.* It may also provide an interesting model for future study of oncogene regulation since the microenvironment of the thymus may somehow

regulate the degree of amplification or mRNA expression and thus regulate cell proliferation.

TcR Expression by TBLs. TBLs were originally isolated as Thy-1^+, Lyt-1^+ cells that lose Thy-1 expression in culture. For this reason, we assumed that these isolates may be of lymphoid origin. With this in mind, we decided to analyze several clones for the rearrangement and expression of the T cell antigen receptor genes. Figure 6 shows the results of Southern blot analysis of the γ and β and δ genes. Figure 6A shows that TBLs rearrange the γ gene and those rearrangements found are identical in each clone. The results of the β gene analysis (Fig. 6B&C), after digestion with four restriction enzymes (PvuII, BamHI, EcoRI and HindIII) in three separate experiments (The EcoRI and HindIII were used together), show no rearrangements. Figure 6 D shows that the δ gene is deleted in all of the TBL lines tested, as expected since a full length α chain mRNA is produced. When the α gene is rearranged, the δ gene is deleted, thus giving no signal on Southern blots (The α chain gene cannot be analyzed using Southern blots because the gene is too large). Northern blot analysis (Fig. 7) revealed identical results in all lines tested. Clearly these cells make a mRNA transcript for each of the genes analyzed, but the pattern in which these genes were expressed was unique. A full length α gene transcript (Fig. 7a) was detected, but it is unlikely that TBLs produce a functional α/β receptor in the absence of a mature 1.3 kb β mRNA (Fig. 7b) (46, 47). The non-productive 1.0 kb β mRNA was produced in these cells. The presence of the short β mRNA in the absence of DNA rearrangement is not unusual. The expression of this 1.0 kb mRNA has been shown to occur only with D-J rearrangement (48, 49) or with no

rearrangement at all (50). Since we could not find evidence of the expression of a functional α/β receptor, we thought possibly this cell line represented an early developmental thymocyte subset that expressed the CD3 γ/δ (51) type receptor. Upon close examination of the blot exposed to the Cγ probe, we discovered that TBLs consistently produced γ chain transcript that is shorter than normal (1.5 kb) (Fig. 7c). We have not yet determined the nature of this shortened transcript. No δ chain transcript was detected in any of the TBL lines (Fig. 7d), consistent with its deletion from the genome observed in the Southern blot results. This pattern of TCR expression suggests that TBLs may represent an aberrant phenotype because no TCR can be produced. On the other hand, these results may represent incomplete rearrangement due to a loss of RAG gene function resulting from immortalization.

The above studies describe a previously unreported T cell phenotype. The cells express neither CD4 nor CD8, and thus can be considered to be immature. As a matter of fact, the only T cell specific cell surface marker expressed by these cells is Lyt-1. They also do not express cell surface TCR, and the genetic expression pattern for this receptor complex will not produce a functional TCR. However, this cell line was shown to bind a characteristic epithelial cell in a monolayer of thymic derived cells. Using the binding potential between these two cell types, we have been able to show these stromal components to be TNCs. In the next section, we show how we chose to further study this interaction.

Characterization of SVT-MP5

Isolation and Immortalization. The cells isolated from fetal calf serum gradients, shown in Fig. 8A, display the characteristic multicellular

complex of TNCs. After 24-48 hours in tissue culture, these cells release their thymocytes and form monolayers (Fig. 8B), but proliferate only to a limited extent (Fig. 9A). To immortalize TNCs, we infected nurse cells isolated from 2 week old C57BL/6 mice with SV40. The size and morphology of the cells obtained post infection (Fig. 8C), flat with an expansive cytoplasm containing cable-like striations, are similar to TNCs found in fresh isolates (Fig. 8B). Cells taken from the log phase of growth were stained with a polyclonal antibody made against the large T antigen, the SV40 transforming protein. Nuclear staining was readily visible in some cells (Fig. 8D), but other cells in the same culture displayed cytoplasmic staining with bright granules centered in the nucleus (Fig. 8E). This type of staining pattern may represent the plasma membrane-associated large T antigen fraction (52). The immortalization of human and rodent epithelial cells by SV40 has been well documented (53, 54). SV40-transformed lines of TNCs have been obtained from each of 11 infections carried out in our laboratory. Infected cells were cloned using limiting dilution. A total of 18 clones was isolated from our original infection. Three of these clones have been maintained in culture for over six months. The other lines were stored in liquid nitrogen.

Increased growth of SV40-infected TNCs above that for uninfected cells was apparent from 10-14 days post infection, at which time they began to double every 18-20 hours (Fig. 9A). Rapid proliferation and the nuclear expression of large T antigen are characteristics of SV40-infected cells, but stable transformation requires the insertion of the SV40 genome into the genome of the host cell. We tested for viral insertion in one of the cloned lines, designated SVT-Mp5, using Southern blot analysis. High molecular weight DNA isolated from SVT-Mp5 was digested with several restriction

enzymes that do not cut within the SV40 genome. The digested DNA was separated on an agarose gel and blotted onto nitrocellulose before exposure to the entire SV40 genome labelled with ^{32}P as a probe (Fig. 9B). Multiple fragments of varying sizes containing SV40 sequences were detected with each enzyme used. The fact that several of the fragments are larger than genomic SV40 DNA verifies viral insertion because they could only be generated by cutting mouse genomic sequences. Large and small fragments were detected upon digestion with EcoRI or BamHI because one restriction site for each enzyme exists within the SV40 genome.

Identification of cells as TNCs. Because TNC-specific antibodies have not yet been developed, we stained each cell line with a panel of antibodies to verify their identity. The data presented in figure 10 are the results of the analyses of SVT-Mp5. A2B5, a neuron-specific antibody that recognizes only TNCs in the thymus (55, 56), was used as an initial screen for each clone isolated. Both normal TNCs and SVT-Mp5 were found to express the A2B5 specific antigen (Fig. 10A and B). The staining pattern obtained using anti-A2B5 suggests that the antigen recognized by this antibody may be associated with stress fibers in the cell. Also, extensive cytokeratin staining characteristic of normal TNCs (Fig. 10C) (57) was detected in SVT-Mp5 (Fig. 10D). SVT-Mp5 cells stained with antibodies made against the major histocompatibility complex class I (Fig. 10F) and class II antigens (Fig. 10H), but were Thy-1 and Mac-1 negative (Table 3). No phenotypic changes have been detected in SVT-Mp5 cells after six months in culture. Together these data provide convincing evidence that the cells described above are thymic nurse cells transformed with SV40.

TNC Internalization of Thymocytes. Although TNCs have the ability to present antigen *in vitro*, their function in the thymus has not been established. The unique sequestering of T cells into specialized cytoplasmic vacuoles, whose membranes contain both class I and class II antigens suggests that information about self may be available to the trapped thymocytes. But the mechanics of this interaction, the T cell subpopulations involved, and the mechanism of their uptake remain unknown. No report of the actual internalization of thymocytes has been published to date. Earlier studies showed nurse cells to bind lymphoblasts isolated from 15 day old embryos (58), forming a unique complex that completely covers the surface of the TNC. In an attempt to test the binding capacity of our SVT-Mp5 cell line, and to characterize the thymocyte subpopulation(s) involved in binding TNCs, freshly isolated thymocytes and cells from several T cell lines were labelled with ^{35}S-methionine and co-cultivated with SVT-Mp5 cells. Freshly isolated thymocytes showed binding levels that were 4-5 times higher than control samples, but the cell line TBL-1 displayed the highest binding capacity (Fig. 11). Adherence of these cells to uninfected TNCs and SVT-Mp5 cells was 8-10 times higher than any other combination of cells. Apparently this interaction was specific because TBL-1 binding was not detected with any other adherent cell type, including those obtained from TNC-depleted thymic preparations. The characteristics of the co-cultivation of TBL-1 cells with SVT-Mp5 are shown in Fig. 12A-G. Within 24 hours (Fig. 12A and B), the lymphocytes completely cover the surface of the nurse cells, and a subpopulation of the TNCs internalize the bound lymphocytes (Fig. 12D-G). Captured lymphocytes can be seen in vacuoles in the cytoplasm of SVT-Mp5 cells. Figures 12E-G show micrographs of the same TNC at

increasing magnifications. At the highest magnification (Fig. 12G), the plasma membrane (arrow) of the SVT-Mp5 cell can be seen covering internalized lymphocytes.

DISCUSSION

This study describes the development of a model system to study the role of thymic nurse cells in T cell differentiation. The study began with the isolation of novel thymus derived cell lines (TBLs). The cells occur spontaneously under simple culture conditions and develop into stable continuous cell lines. Preliminary experiments of their growth characteristics show these cells to grow rapidly in tissue culture but are non-tumorigenic when injected intrathymically or intraperitoneally into an adult congenic host (table 2). Two months after transplantation the cells remain in the thymus. This suggests that TBLs are a very immature phenotype, because they still recognize the signals or factors which allow them to home to the thymus, much the way bone marrow derived T cell precursors are thought to, during the colonization of the fetal thymus. Since TBLs do not cause thymic tumors, we believe that there must be something about the microenvironment of the thymus which controls their growth *in vivo*. How this thymic homing and growth control is mediated is unknown, but we might speculate that it involves interactions with thymic stromal cells, possibly TNCs, in light of TBLs specific interaction with both fresh and SV40 immortalized TNCs in culture. One thymic epithelial cell line (IT-76M1) was shown to stimulate thymocyte proliferation through the secretion of thymic hormones (59). We might speculate that if some factors released by thymic epithelial cells can stimulate proliferation, others may inhibit it. From a functional point

of view, TNCs were shown to secrete thymic hormones (60) and promote phenotypic changes on maturing thymocytes (33). In addition to TNCs, another thymic epithelial cell line was shown to secrete a polypeptide able to attract precursors into the thymus (61, 62). Could TNCs be responsible for the homing of TBLs to the thymus? Could they regulate their growth *in vivo* ? We have shown that TBLs have an increased expression of the *c-myc* gene from an amplified genome (Fig. 4 and 5). The B-Ly1 cell line was also shown to have elevated *c-myc* expression as a result of amplification of the *myc* gene, without evidence of local DNA rearrangement (63). In lymphoid neoplasia, *c-myc* deregulation has been related to local proviral insertion (64, 65), or translocation to an immunoglobulin locus (66, 67), thus *c-myc* amplification in B-Ly1 cells and TBLs is a unique mode of *myc* activation in the lymphoid lineage. Various researchers have questioned how the B-Ly1 cell line acquires the Ly1 marker, since it is a B cell and Ly1 is normally expressed on T cells. We could speculate that TBLs represent an early developmental step in the development of the B-ly1 lineage, which acquires Ly1 expression during its passage through the thymus on the way to the spleen. It should be noted however that TBLs do not express IgM on their cell surface (Table 1),while B-ly1 cells do. In addition, we have analyzed the state of the TCR genes in B-Ly1 cells and found that they are in the germ line configuration. It seems unlikely then, that these two cell lines are related.

Spontaneous gene amplification is a common occurrence in most cell types (68), and in the case of B-Ly1 cells and TBLs may simply reflect the outcome of selection for the capacity of *in vitro* growth. However, it seems odd that this growth would occur by the same mechanism, in rather rare cell types, in two separate systems. These data may indicate that the *in vitro*

absence of some growth controlling element(s) present in the intact thymus and spleen is responsible for the spontaneous growth of these two cell lines in culture.

The expression of cell surface Lyt-1 by TBLs in the absence of other T-cell specific antigens (Thy-1, LyT2 (CD8), L3T4 (CD4)) is similar to a phenotype isolated from mixed leukocyte cultures by Goodwin et al. (69). This line originated as a Thy-1^+ clone but lost Thy-1 expression in continuous growth cultures, as do TBLs. However, unlike TBLs, the cell line developed by Goodwin et al. was reported to have the ability to repopulate the thymus *in vivo*. When TBLs were analyzed for expression of CD4 or CD8 after intrathymic injection, no mature phenotypes were found. Thus, they should not have the ability to repopulate the thymus. BLy1 cells are another Thy-1^-, Lyt-1^+ lymphocyte subset, but because they express certain B cell marker such as C3, the Fc receptor and cell surface IgM, they are believed to represent a diversion from the normal B cell linage, and not of the thymocyte lineage. Greenberg et al. (70) have isolated a series of spontaneous lymphomas from AKR mice that are Thy-1^+, Lyt-1^+ but express the B cell marker IgM. Likewise, Lanier et al. isolated Lyt-1^+ lymphomas that expressed different Ig isotypes. Future studies of TBLs and the other cell lines mentioned above may yield some insight to the relationship of these distinct Lyt-1 bearing populations.

The rearrangment of T cell antigen receptor genes distinguishes TBLs as thymus derived cells, although their expression pattern is unusual. The normal sequential expression pattern of the TcR complex genes during early thymocyte development has been reported to be γ first, followed by β then α- mRNA (71). The cells reported here clearly produce a full length α- chain mRNA and a truncated γ-chain mRNA in the

absence of a full length β chain message. No δ chain mRNA is produced, as expected since the δ gene is deleted when the α gene is rearranged. TBLs occur early in the embryonic thymus (day 13 to 14) and may respond to signals for the rearrangement and expression of the TcR genes. However, they are probably unable to produce either an α/β or a γ/δ type receptor and may represent an aberrant cell type. With this we believe that we have isolated a previously unreported T cell line with immature characteristics. Whether TBLs have some function in the mouse, or represent an intermediate in some as yet undiscovered developmental pathway remains a mystery. They are similar to B-Ly1 cells, but it is clear that they are a unique cell line, because they rearrange the TCR and do not express IgM. TBLs along with B-Ly1 cells represent an interesting system to study the regulation of *c-myc* oncogene expression. The most interesting and significant characteristic of TBLs, however, is their specific interaction with TNCs.

Thymic epithelial cells are believed to play a major role in T lymphocyte development (72, 73). Long-term thymic epithelial cell lines have been established recently to study their functional activity on thymocytes *in vitro* (74). One such line was shown to bind immature T lymphocytes in cultures from both mice (75) and humans (76). Another cell line produces factors capable of modulating thymocyte response to T cell mitogens (77). Regardless of the unique characteristics of subpopulations found within the thymic epithelium, most grow in monolayers *in vitro* and express cytokeratins. TBLs have been shown to bind specifically to thymic nurse cells. Forty eight hours later the TBLs completely cover the attached stromal cells. Physical removal from their attachment to the TNCs results in the formation of large sheets of

suspended cells. Although TBLs may offer little potential for the study of various steps in T cell development, their strong, specific interaction with thymic nurse cells makes them a useful counterpart in a model system to study the binding and internalization process utilized by thymic nurse cells in the thymus. By generating monoclonal antibodies to both TBLs and TNCs, we should be able to produce antibodies specific for the proteins involved in the specific internalization of lymphocytes by TNCs. These antibodies could be used to both study the function of the internalization process, and to isolate the proteins involved. These isolated proteins could then be sequenced to allow the generation of probes for cDNA cloning.

In order to develop a model system which might ultimately enable the determination of the role of TNCs in T cell development, we tried to produce a cell line which maintains the ability to bind and internalize lymphocytes in culture. By developing a method to infect freshly isolated TNCs with SV40, we have been able to develop many TNC lines, including one, SVTNC MP5, which retains that ability. Thymic nurse cells were discovered in 1980 (21). Since that time it has been difficult to investigate their function because methods for their isolation yielded insufficient numbers of cells to study. Ezaki et al. reported that the spontaneous thymomas which develop in BUF/Mna rats are highly enriched for TNCs, while the structure of the thymus, although greatly enlarged, remains very similar to the normal thymus in control animals (42). This system will certainly enable the isolation of larger numbers of TNCs, but it still possesses many of the same restrictions encountered when trying to use fresh cells from normal animals. There will still be contaminating lymphocytes, and the cells isolated from the thymomas

have a very limited growth potential. Also, any conclusions drawn about specific functions of TNCs, implied by results obtained with this model system, will be subject to the same criticisms proposed about other organ culture, and *in vivo* systems. The complexity of the system will prevent the direct application of particular functions to TNCs, while ruling out the necessity of other cell types. Only one TNC line has been reported to date (58). Its clone was established from a spontaneous C57BL/6 thymic tumor. Although this line was demonstrated to bind lymphocytes in culture, no internalization was reported nor was the type of thymocytes described. Unfortunately, these may or may not be TNCs. They could represent another type of thymic epithelial cell, such as dendritic epithelial cells which are also reported to bind lymphocytes *in vivo*. We report here an easy *in vitro* method of immortalizing TNCs with SV40. SV40 has been used previously to transform low density thymic epithelial cells (78). The cells described in that study were small (35-50 mm) keratin-expressing cells with large nuclei. In this report the cells resulting from SV40 infection were very large (80-100 mm; Fig. 12), and maintained many of the characteristics of freshly-isolated TNCs. One line retained the ability to internalize thymocytes. This is the first report of the actual internalization of thymocytes by TNCs. In fact, every study reported to date on TNC function or TNC thymocyte development was facilitated through the use of freshly isolated TNCs containing cytoplasmic thymocytes. This was the only way the TNC lymphoepithelial complex could be identified. Further, freshly isolated TNCs do not take up thymocytes in culture (79). We propose that many of the interactions between TNCs and their internalized thymocytes exist before the characteristic multicellular complex can be detected in the normal thymus. The system described here

will allow for a detailed *in vitro* analysis of the relationship between the two cell types in future investigations. The development of a TNC line with internalization capabilities was important because now many important questions about the function of TNCs in T cell differentiation can be asked. What stage of T cell development requires passage through the TNC? Are any cells released from TNCs, and if so what are the phenotypes of the released cells? What is the specific role of self antigen presentation by TNCs in T cell education? The TNC lines obtained have been maintained in continuous cell culture. They have been passaged twice a week for over one year. Even if these cells prove to be unstable in tissue culture with time, we have repeatedly obtained infection and transformation of TNCs with SV40, which resulted in sufficient numbers of cells to study. In addition, we have recently been able to produce temperature-sensitive lines using the tsA58 virus, a mutant form of SV40. These lines may prove even more useful, as they may represent a more normal state at the nonpermissive temperature.

The definitive identification of TNC T cell subpopulations is crucial to the determination of nurse cell function. Both immature and mature T cell phenotypes have been found within TNCs (80), but the majority were found to be nonfunctional. The major problem with previous studies of TNC thymocytes was the inability to completely separate them from the general thymus T cell population. This, along with the fact that the TNC thymocyte population is heterogeneous has made it very difficult to obtain definitive information about these cells. We are currently analyzing the normal (isolated from fresh thymus tissue) T cell population internalized by SVT-Mp5 using a panel of monoclonal antibodies that recognize T cell specific surface markers in *in situ* staining experiments. These experiments

have shown that the thymocyte subset which is bound and internalized by our SV40 immortalized cell lines is a subpopulation of the double positive phenotype. These lymphocytes express CD4, CD8, CD3 and the α/β TCR, making them mature double positives, which are thought to be the precursor to single positive functional cells (Cellular Immunology 1991,in press). Immunofluorescence staining of those thymocytes released from SVT-Mp5 cells may provide insight about the T cell specific changes that occur within the TNC cytoplasm. Finally, our TNC lines are being used to produce TNC-specific antibodies for use in our binding studies in an attempt to define the protein(s) involved in the internalization process. Since the experimental evidence suggests that it is the thymic stroma which is responsible for positive selection (15), TNCs are a likely candidate for this function. TNCs have been shown to express MHC class I and class II antigens on their surface, and on the inside of the specialized vacuoles which surround internalized lymphocytes(21, 33) They have also been shown to be able to present self antigens to T cells (26). By using a TNC-specific monoclonal antibody we may be able to produce a mouse which is lacking thymic nurse cells, or simply block their functional receptors,as was done with CD4, CD8 and MHC antibodies (11, 12). Then, by using a system similar to the Vβ17a model developed by Kappler et al. (9), we should be able to determine a specific role for TNCs. A more direct use of the TNC lines we have developed could involve *in vitro* organ culture or simply our TNC lines with TNC-depleted monolayers as feeders. Thymic nurse cells may require additional factors supplied by other thymic stromal cell types in order to function. These would be supplied by the feeder layers, or by the microenvironment of the thymus in organ culture experiments. In this type of experiment we would have much more

control over the lymphocyte subpopulations added as well as the types of thymic feeder cells. We could then identify any changes in the cell surface phenotype exhibited by lymphocytes after interacting with TNCs. The diversity of cell types shown, by other groups, to be present in thymic nurse cells *in vivo* suggests that differentiation may be occuring within this unique microenvironment (33, 34). Finally, some groups have suggested that the cortical epithelial cells involved in positive selection may somehow make lymphocytes insensitive to the corticosteroids, which are thought to induce apoptosis in the unselected T cells (14, 18, 33). Through the use of markers that distinguish normal from apoptotic cells, such as toluidine blue (81), we may be able to shed some light on this topic. We could allow our TNC lines to internalize lymphocytes, and then determine if apoptosis has been induced. The most recent data to come from our studies of these SV40 immortalized cell lines has been obtained by filming live cells, which had been mixed with lymphocytes, using Hoffman contrast microscopy. Using this technique we have been able to document both the internalization process and the release of internalized lymphocytes by live TNCs in culture (unpublished data). It will be interesting to determine the fate of these released lymphocytes. We believe the system described here will provide a practical method to study the role of TNCs in T cell education.

Table 1 Cell Surface Characteristics for Various TBL Clones

	Antibodies											
Cell Line	Thy 1	Lyt-1	CD4	CD8	IL-2r	H-2	IA	IgM	Ly-5	Mac-1	TCR	IgD
TBL-1	-	+	-	-	-	-	-	-	-	-	-	-
TBL-2	-	+	-	-	-	-	-	-	-	-	-	-
TBL-3	-	+	-	-	-	-	-	-	-	-	-	-
TBL-7.5	-	+	-	-	-	-	-	-	-	-	-	-

The cells that were originally cloned as Thy-1+ cells (Fig 1) were grown to confluency and reanalyzed with the antibodies shown above. Positive staining was determined using FACS analysis.

Table 2 The Homing Ability and Tumorigenicity of TBL-1

Intrathymic Injection			Intraperitoneal Injection		
Expt.	Cell count X 10^4	%Lyt-1.2$^+$	Expt.	Cell Count X 10^4	%Lyt-1.2$^+$
1	8.3	0.46	1	9.2	0.80
2	10.4	0.20	2	1.2	0.53
3	8.4	0.60	3	5.2	0.23
4	4.9	0.47	4	1.2	0.75

Table 3 Characteristics of the SV40-Transformed TNC Line SVT-MP5

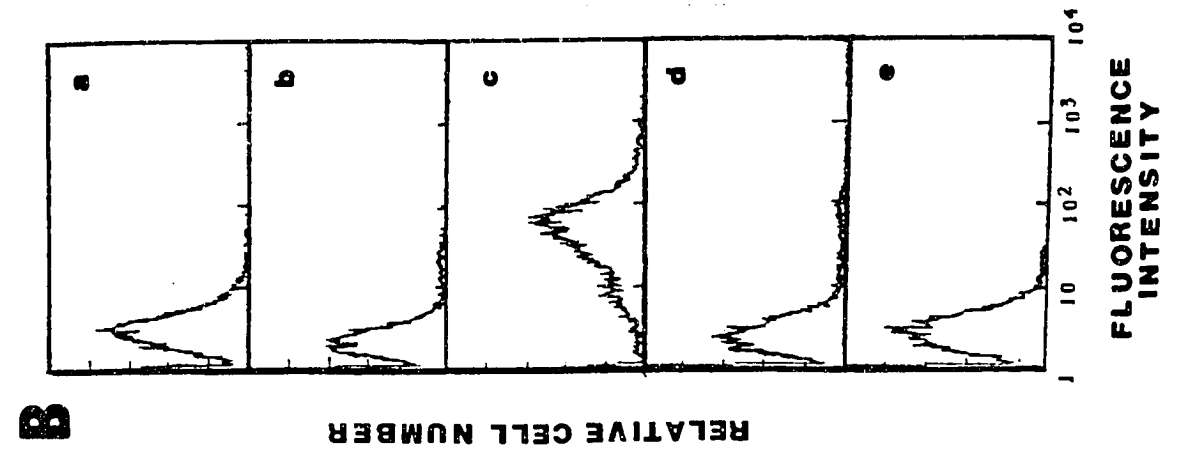
	Antibodies							
	T ag[a]	A2B5	Keratin	Ia[b]	H2b	Mac-1	Thy-1	Internalization
TNCs (uninfected)	-	+	+	+	+	-	-	-
SVT-MP5	+	+	+	+	+	-	-	+
TNC-depleted thymic stromal cells[b]	-	-	+	+-	+-	-	-	-

Cells were stained with the appropriate primary antibody plus a fluorescent secondary antibody and visualized using fluorescence microscopy. + means that staining was obtained. - means no staining was obtained.
+- most stained some not stained.

[a] Anti-T antigen antibody

[b] The procedure used to isolate TNCs was exploited here to obtain thymic stromal cells devoid of nurse cells (See Materials and Methods section). TNCs form pellets in fetal calf serum gradients, but the remaining cells remain suspended in the medium. This step was performed twice and all of the cells at or above the FCS layer were collected and plated in tissue culture dishes. Those cells that form monolayers were considered to be TNC-depleted thymic stromal cells.

Figure 1. Isolation and cloning of TBL-1. Thymi from 14-15 day old C57BL/6 fetal pups were dispersed into multi-well plates. 10-14 days later individual colonies were isolated and grown to confluency in T-25 flasks. (A) Anti-Thy-1 antibody analysis of one of the isolated colonies. $1X10^6$ cells were stained with anti-Thy-1. Thy-1 positive cells (live cells labelled with anti-Thy-1 are shown in quadrant 2) were sorted one cell per well into 96 well plates and grown to confluency. One of the clones, TBL-1, is analyzed in panel B. (B) (a) Fluoresceinated second step anti-mouse IgG alone, (b) anti-Thy-1, (c) anti Ly-1, (d) anti-CD4, or (e) anti-CD8. Analysis of the stained cells was performed using a FACS.



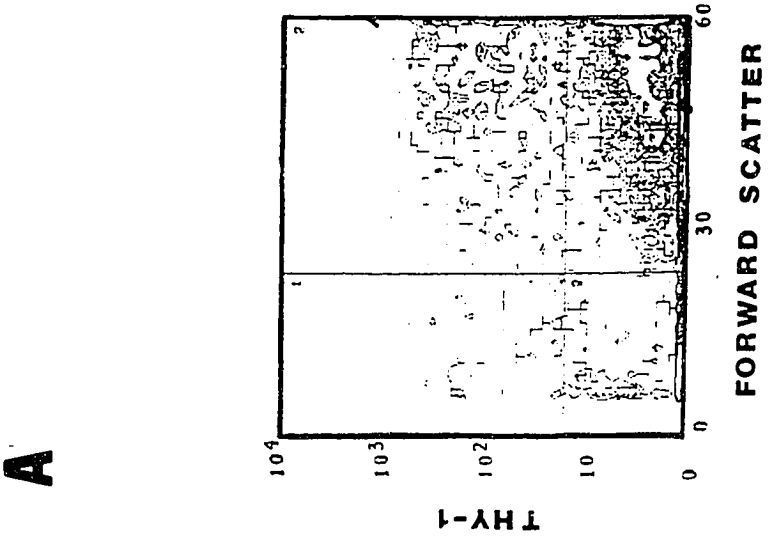


Figure 2A. Fetal calf serum requirements of TBL-1 cells in culture. The cells were seeded at $1x10^4$ cells/well in 6 well plates. Individual wells contained RPMI, gentamycin plus, 10% fetal calf serum (●), 5% fetal calf serum (o), 3% fetal calf serum Δ , 1% fetal calf serum ▲ , and 0% fetal calf serum (■). Each point represents the average cell count of three plates. Viability was determined by cytoplasmic exclusion of Trypan Blue.)) Indicates cell death.

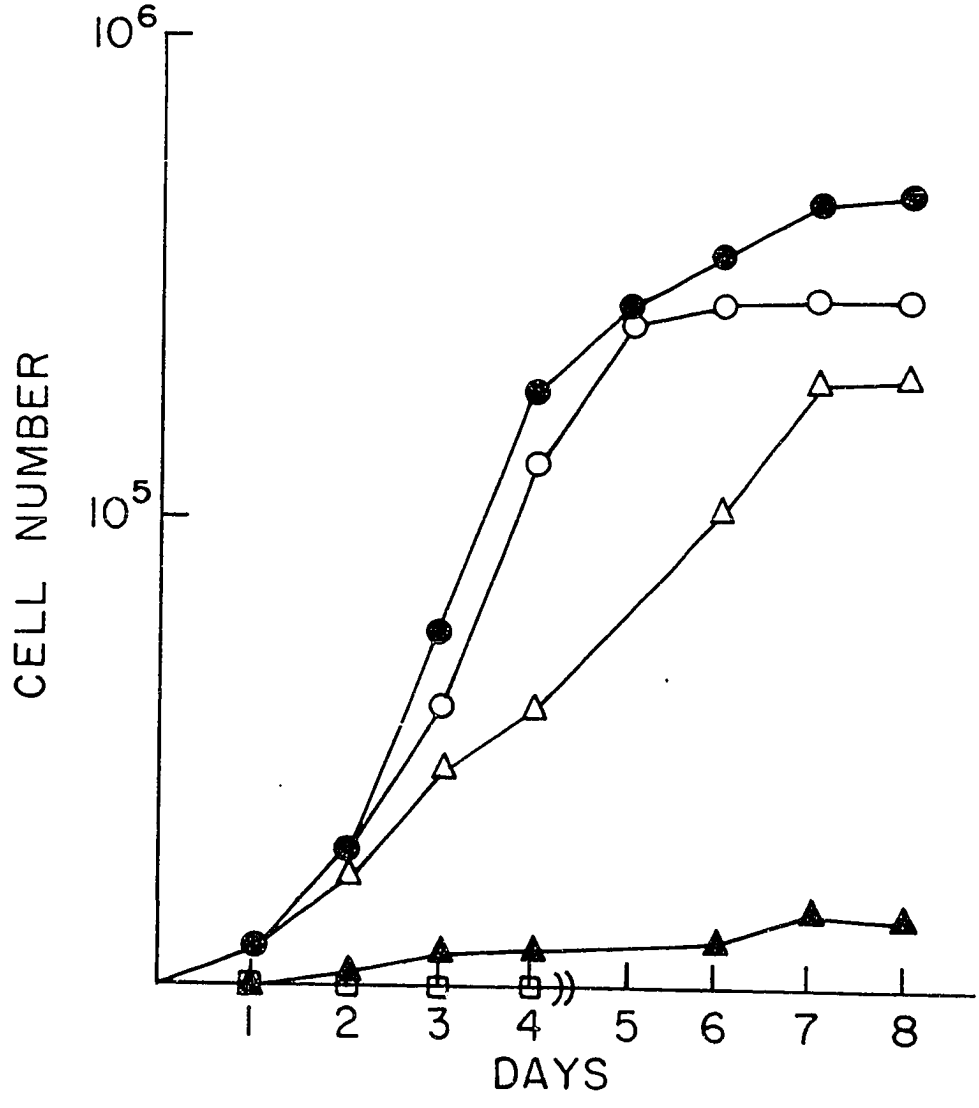


Figure 2B. The effect of Nuserum concentration on TBL-1 growth in culture. TBL-1 cells were seeded at $1x10^4$ cells/well in 6 well plates. Individual wells contained RPMI, gentamycin, 10% fetal calf serum, and 0% Nuserum (o), 1% Nuserum (◘), and 2% Nuserum (●).

The Effect of Nuserum on the Growth of TBL-1

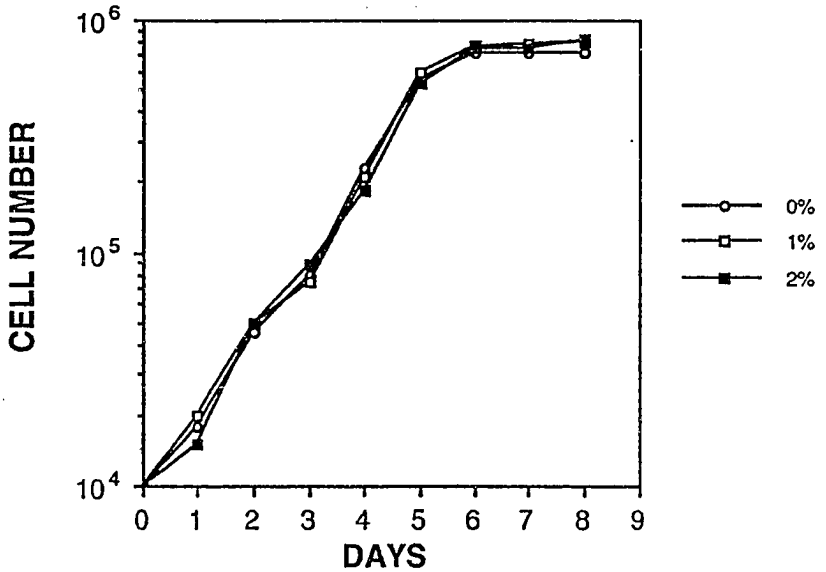


Figure 3. Binding of TBL-1 to freshly isolated TNC in culture. (A) Phase contrast micrograph of TBL-1 in culture (200 x) (B) Freshly isolated TNC after being allowed to adhere to the culture dish and release internalized lymphocytes (200 x). (C) TBL-1 cells bound to fresh TNC after 12 hrs in co-culture (200 x) (D) TBL-1 cells completely covering fresh TNC after 24 hrs. The arrow indicates a small portion of the TNC which is not covered (200 x).

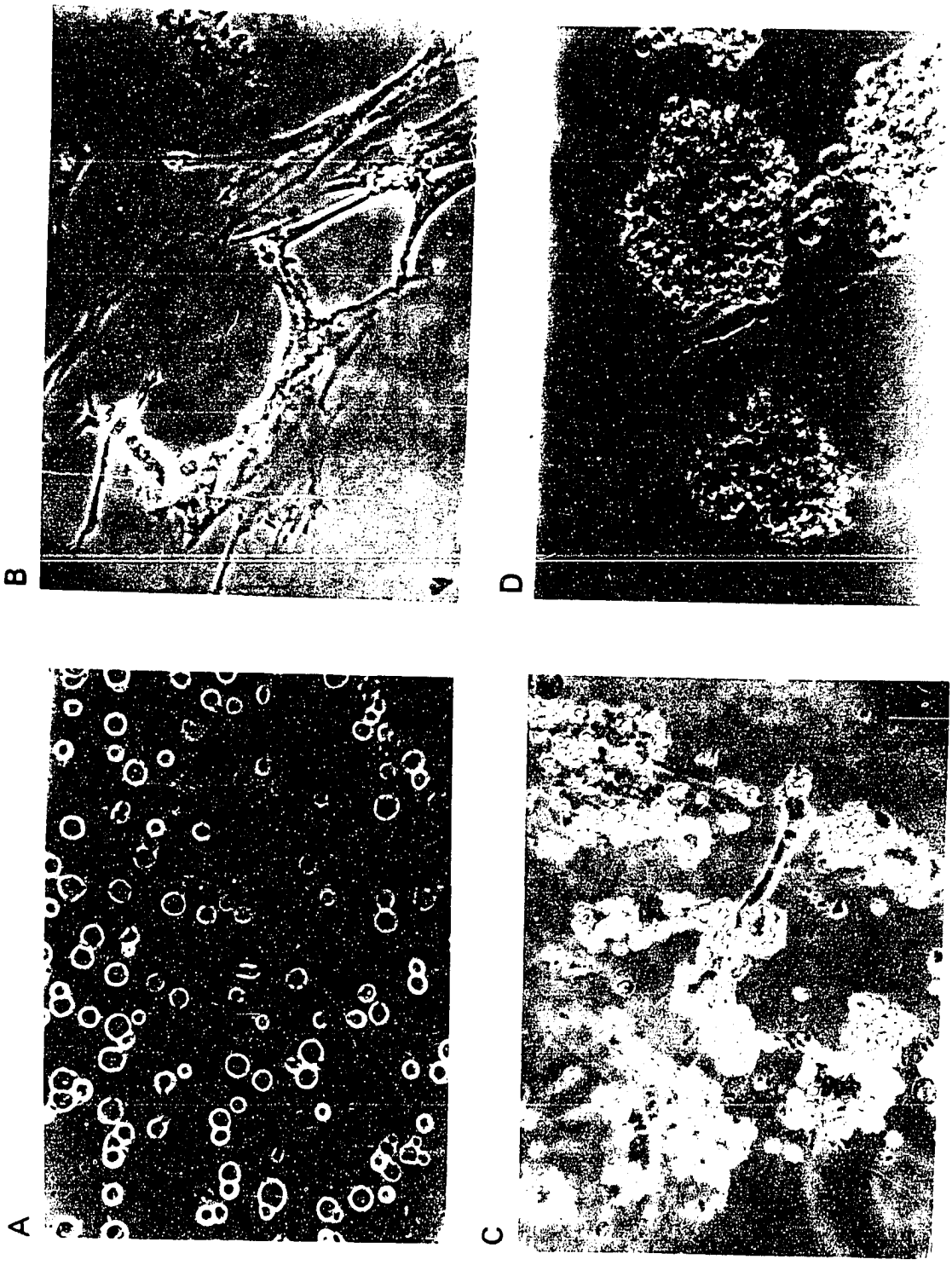
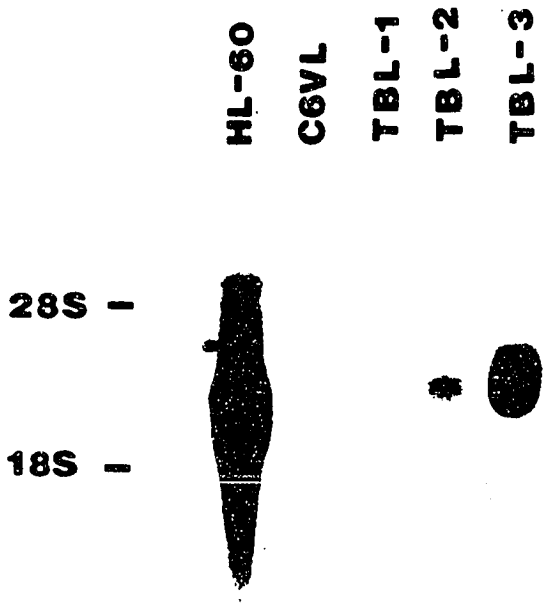


Figure 4. Northern blot analysis of *c-myc* mRNA expression by TBLs. (A) Whole RNA was isolated from HL60 (positive control for over expression), C6VL (T cell, baseline expression), TBL-1, TBL-2, and TBL-3. 10 ug of RNA was loaded per lane, separated by overnight electrophoresis on formaldehyde gels and blotted to nitrocellulose. The blots were then hybridized with a ^{32}P labeled probe cut from the third exon of *c-myc* . 10 to 70 times more hybridization is seen with the TBL lines when compared to the baseline control C6VL. (B) Ethidium bromide staining of the same gel after transfer to show the integrity and quantity of the RNA loaded in each lane.

A



B

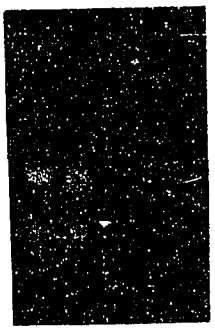


Figure 5. Southern blot analysis to demonstrate *c-myc* amplification in TBLs. 10 μg of DNA was digested for 2 hours with Hind III and BamHI (see brackets). The resulting fragments were separated by overnight electrophoresis on a 0.9% TAE-agarose gel and blotted onto nitrocellulose. Each blot was incubated with ^{32}P- labelled probes specific for *c-myc* and β-actin (single copy control). For each set of lanes, the lower more intensely hybridized bands represent *c-myc* , while the upper less intensely hybridized bands represent β-actin. The number and size of the c-myc hybridizing fragments is the same for both the TBL lines and the controls, showing that there have been no local rearrangements of the DNA around the *c-myc* gene. However, laser densitometry shows that for *c-myc* , 10 to 70 times more hybridization is obtained in the TBL lanes when compared to the liver and spleen controls. Actin hybridization is approximately equal for all lanes.

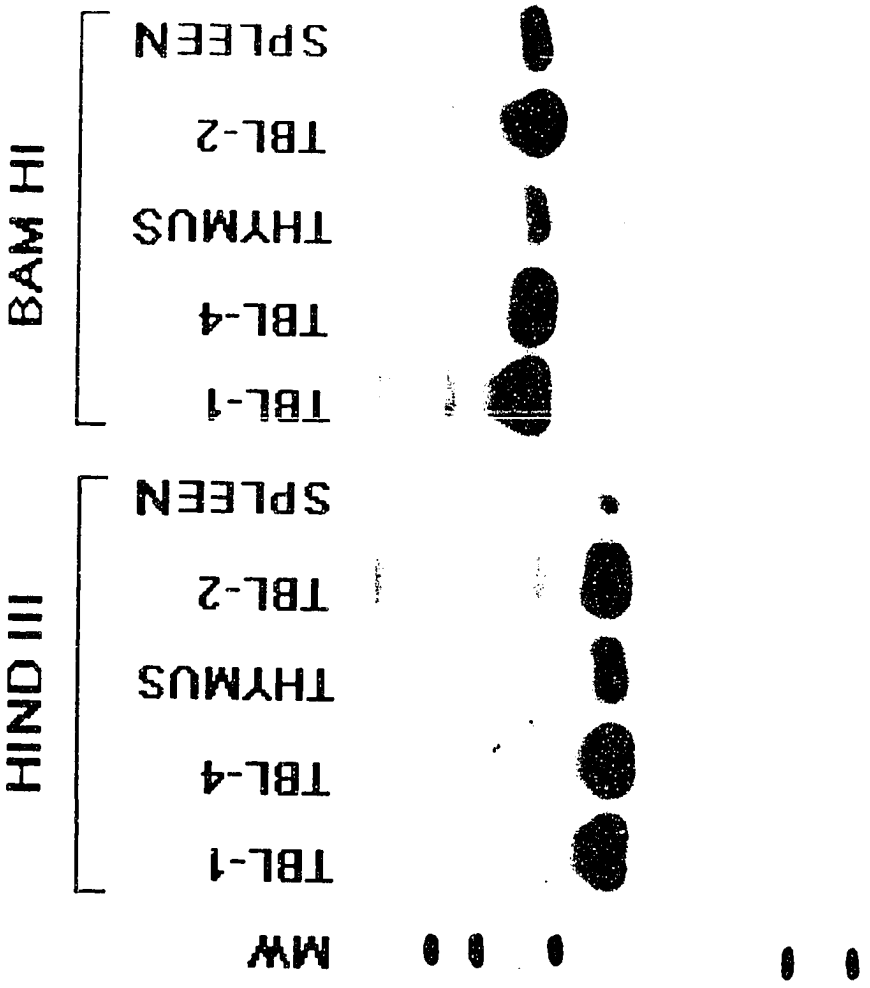
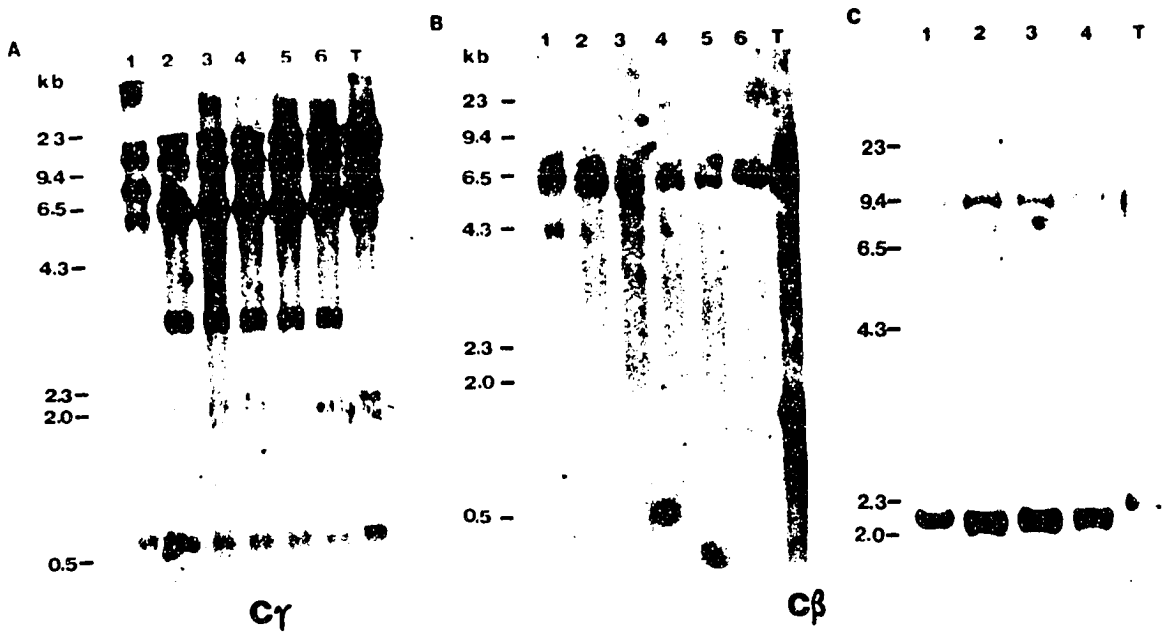


Figure 6. Southern blot analysis of the TCR genes in TBL-1. 10 μg of DNA was digested for 2 hours with (A) Eco RI, (B) Eco RI, (C) Pvu II , (D) BamHI lanes 1-6 Eco RI and HindIII. The resulting fragments were seperated by overnight electrophoresis on a 0.9% TAE-agarose gel and blotted onto nitrocellulose. Each blot was incubated with ^{32}P- labelled probes specific for (A) Cγ or (B and C) Cβ or (D) Cδ. (A and B) Lane 1, C57Bl/6 liver; Lane 2, TBL-1; Lane 3, TBL-2; Lane 4, TBL-3; Lane 5, TBL-4; Lane 6, TBL-5; Lane T, C57Bl/6 thymus. (C) Lane 1, C57Bl/6 liver; Lane 2, TBL-1; Lane 3, TBL-2; Lane 4, TBL-3; Lane T, C57Bl/6 thymus. (D) Lanes 1 and 7 , TBL-2; lanes 2 and 8, TBL-3; lanes 4 and 10, TBL-4; lanes 3 and 9 , C57Bl/6 thymus; lanes 5 and 11 C57Bl/6 liver; lanes 6 and 12, C57Bl/6 spleen.



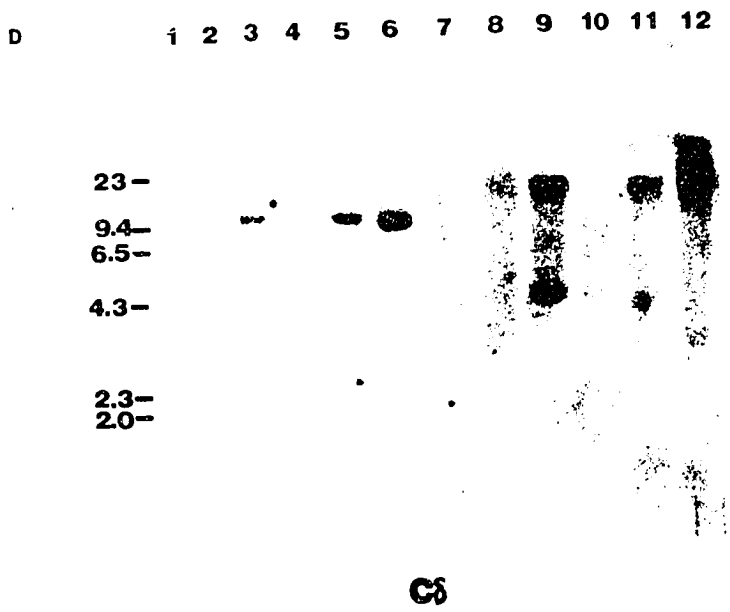


Figure 7. Northern blot analysis of TCR gene expression in TBL-1. 10 μg of whole cell RNA was subjected to electrophoresis on a one percent agarose gel containing 2.2M formaldehyde in morpholinopropanesulfonic acid-acetate-EDTA for 560 volt hours. After transfer to nitrocellulose, each blot was hybridized with ^{32}P-nick translated probe specific for (A) C α; (B) C β; or (C) C γ (D) C δ. (A & B) Lane 1, the T lymphoma line C6VL ; Lane 2 , the B cell myeloma 8653; Lanes 3-5, TBL clones: (C) Lane 1, C6VL; Lane 2, the B cell myeloma 8653; Lane 3-5, TBL clones. (C) the upper arrow shows the size of a normal γ chain mRNA produced in C6VL cells, the lower arrow shows the size of the γ chain mRNA produced in TBL cells: (D) Lane 1, 717 T cell line (δ positive); Lane 2 8653 B cell; lanes 3-5 TBL clones.

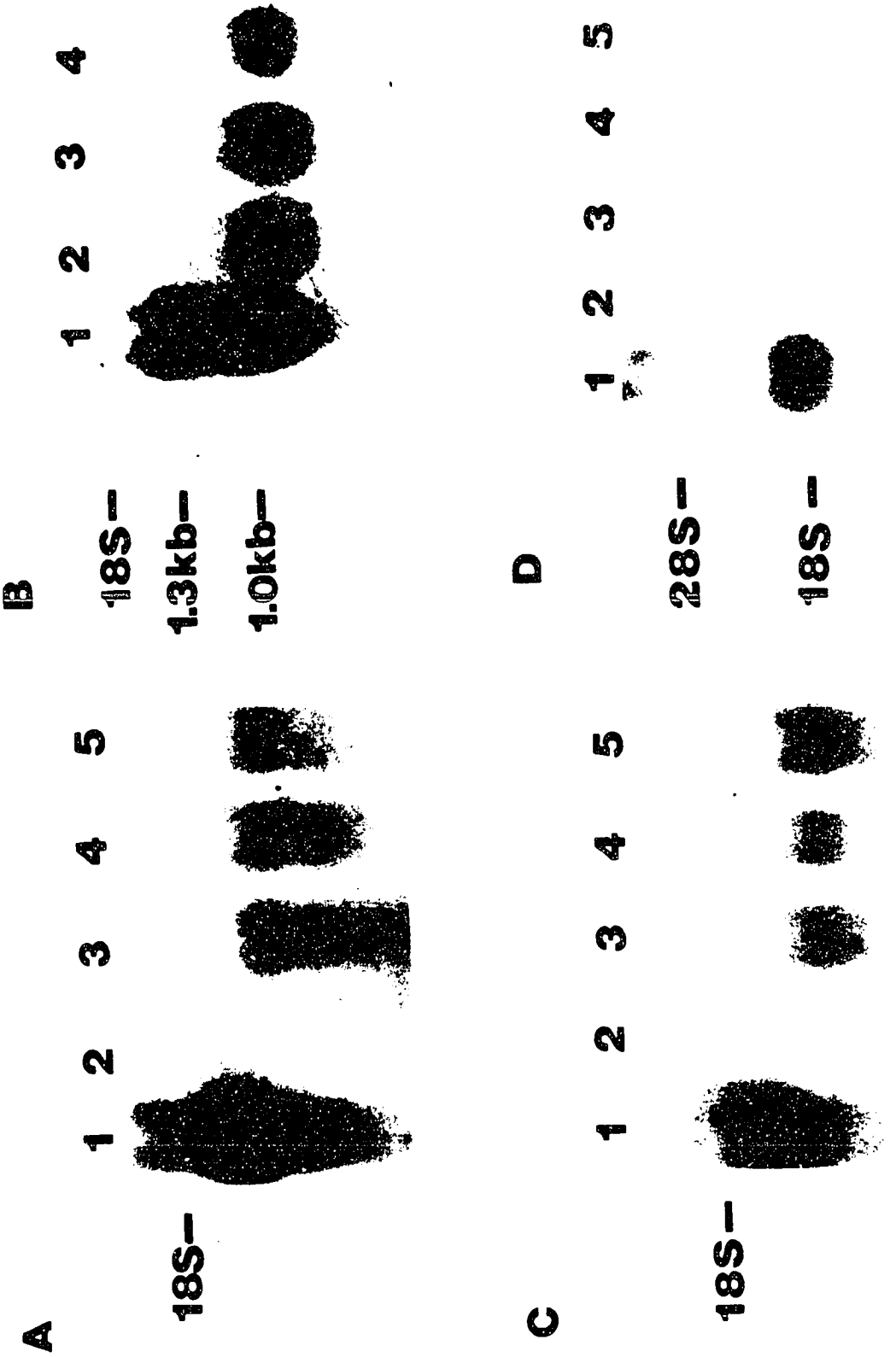


Figure. 8. Isolation and transformation of TNCs. (A) TNCs isolated from the thymus of 2 week old C57BL/6 mice. Magnification 200 X. (B) Isolated TNCs after 24-48 hours in tissue culture. Magnification 400 X. (C) Phase contrast photograph of the SV40-transformed TNC line SVT-Mp5. Magnification 400 X. (D and E) SVT-Mp5 stained with a hamster polyclonal anti-T antigen antibody (provided by Dr. Robert Carroll, New York Medical Center). (F) SVT-Mp5 stained with no first step antibody.

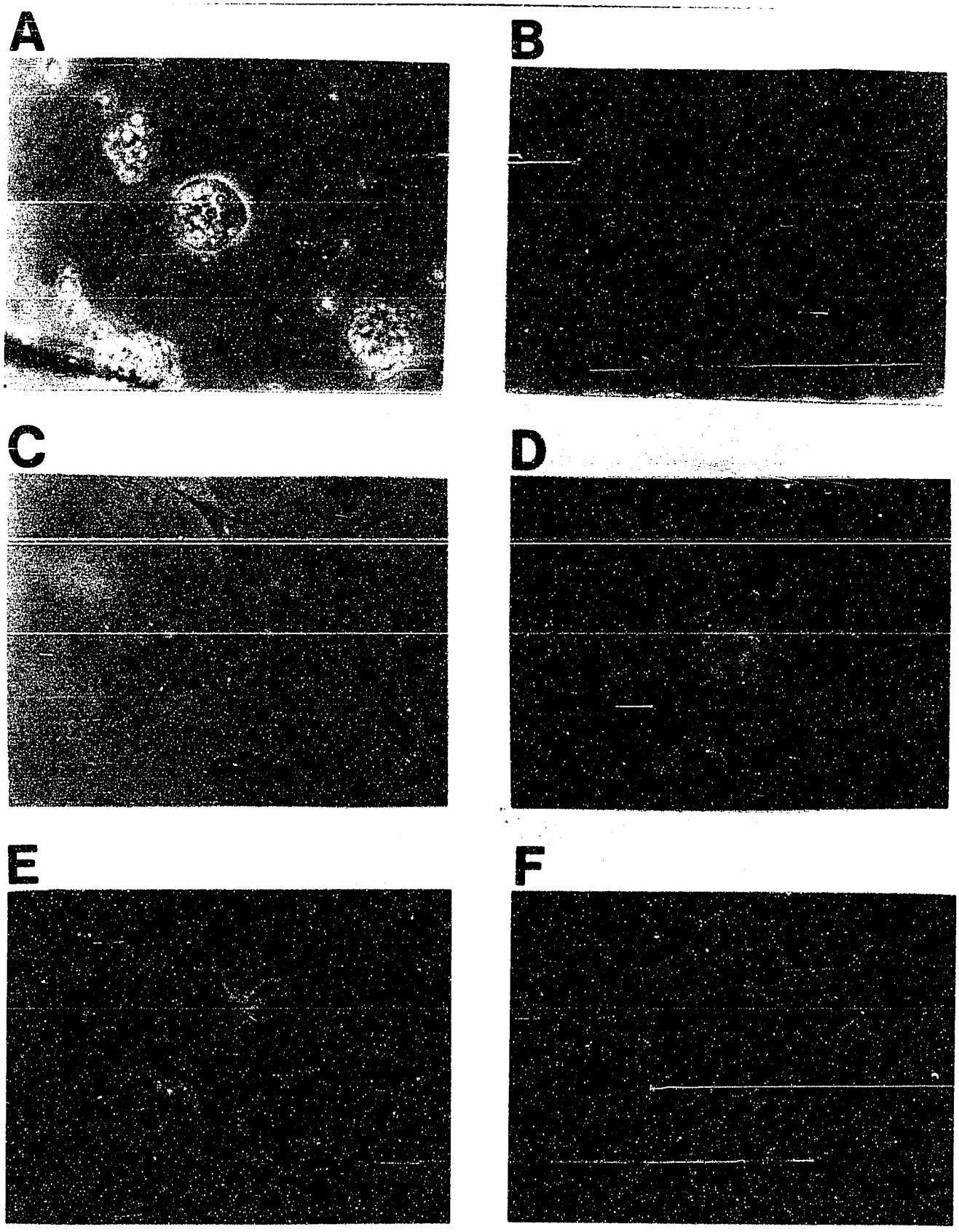


Figure 9. Growth potential of fresh and SV40-infected TNCs. (A) Uninfected and SV40-infected TNCs were seeded at 10^4 cells per 35 mm plate and fed with 3 ml of growth media. Cells were trypsinized and counted at 24 hour intervals. Each value is an average of three plates. Viability was determined by Trypan blue exclusion. (B) Southern blot analysis. The enzymes, AvaI, XbaI, BglII, and SacI do not cut within the SV40 genome. EcoRI and BamHI each has one restriction site within the SV40 genome. High molecular weight DNA isolated from NIH-3T3 cells and digested with EcoRI was used as a negative control. Uncut SV40 DNA was analyzed in the last two lanes, 5 and 30 pg, respectively. The entire SV40 genome was labeled with ^{32}P and used as a probe.

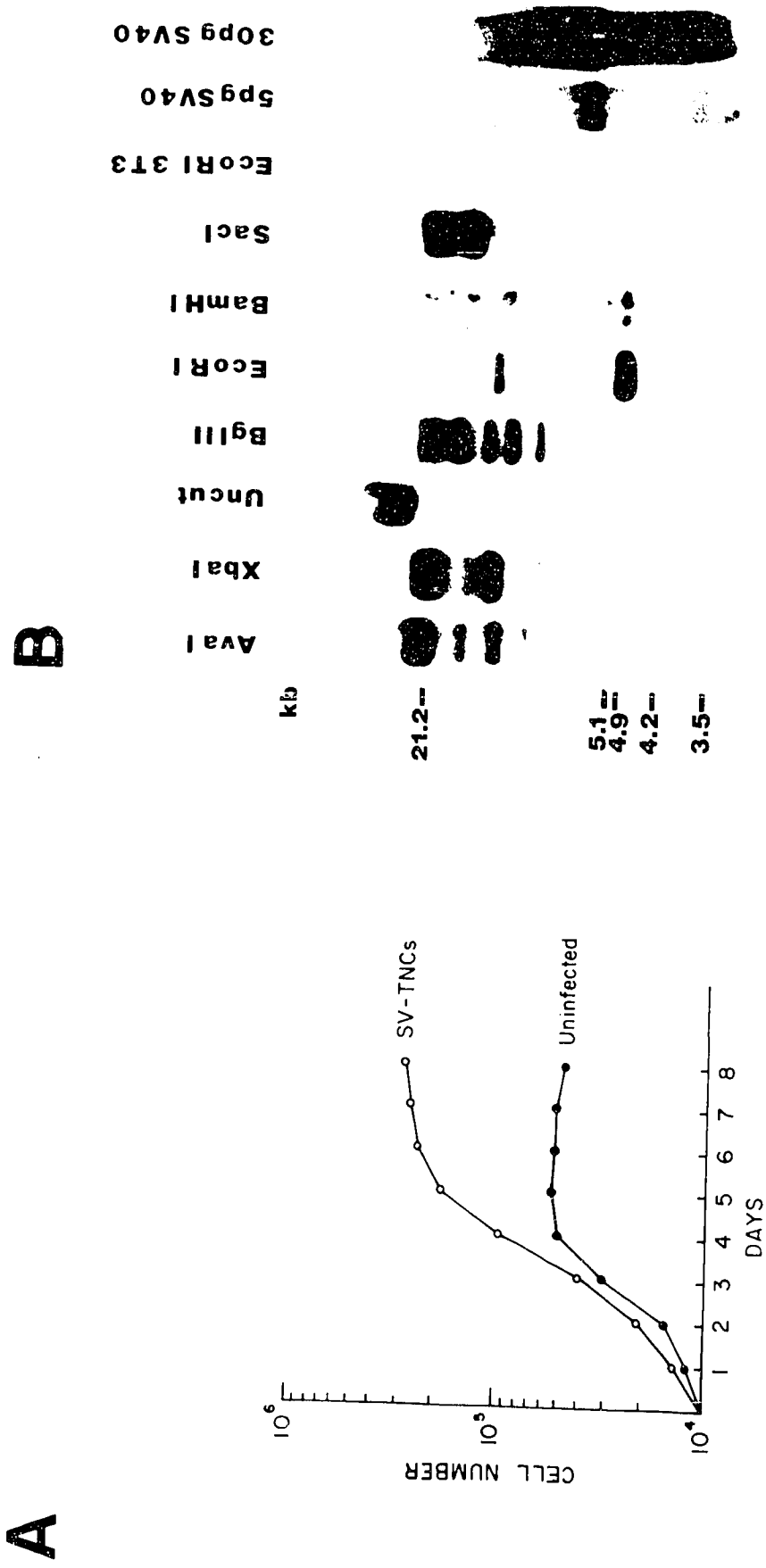


Figure 10. Verification of SVT-Mp5 as transformed TNCs. (A) Freshly isolated TNCs stained with anti-A2B5 (American Type Culture Collection, A2B5 clone 105). (B) SVT-Mp5 stained with anti-A2B5. (C) Freshly isolated TNCs stained with a rabbit polyclonal anti-keratin anitbody (Dr. T.-T. Sun, New York Medical Center). (D) SVT-Mp5 stained with anti-keratin antibody. (E) Freshly isolated TNCs stained with anti-Ia^b. (F) SVT-Mp5 stained with anti-Ia^b antibody. (G) Freshly isolated TNCs stained with anti-$H2^b$ (Accurate Chemical). (H) SVT-Mp5 stained with anti-$H2^b$. No staining was found in samples in which no first step antibody was used.

Figure 11. Binding assay. Each bar graph represents the average of six experiments. C6VL, EL4, and TBL-1 are T cell lines. J558L is a B cell line. Freshly isolated thymocytes and B cells (Spleen) were isolated from the thymus and spleen, respectively, of new born C57BL/6 mice. Each type of lymphocyte was labelled, and incubated with the appropriate monolayer for 16 hours before washing away unbound cells. The percentage of bound lymphocytes was determined as a function of the total number of counts added to each dish. Background level counts were also obtained when the T cell lines BW5147, WEHI-7.1, and WEHI-22.1 were used in the binding assay. TNC-depleted monolayers were obtained by plating thymus derived cells removed from the top of fetal calf serum gradients described in the Materials and Methods section.

Binding Assay to Demonstrate the Specific Interaction of TBLs with TNCs

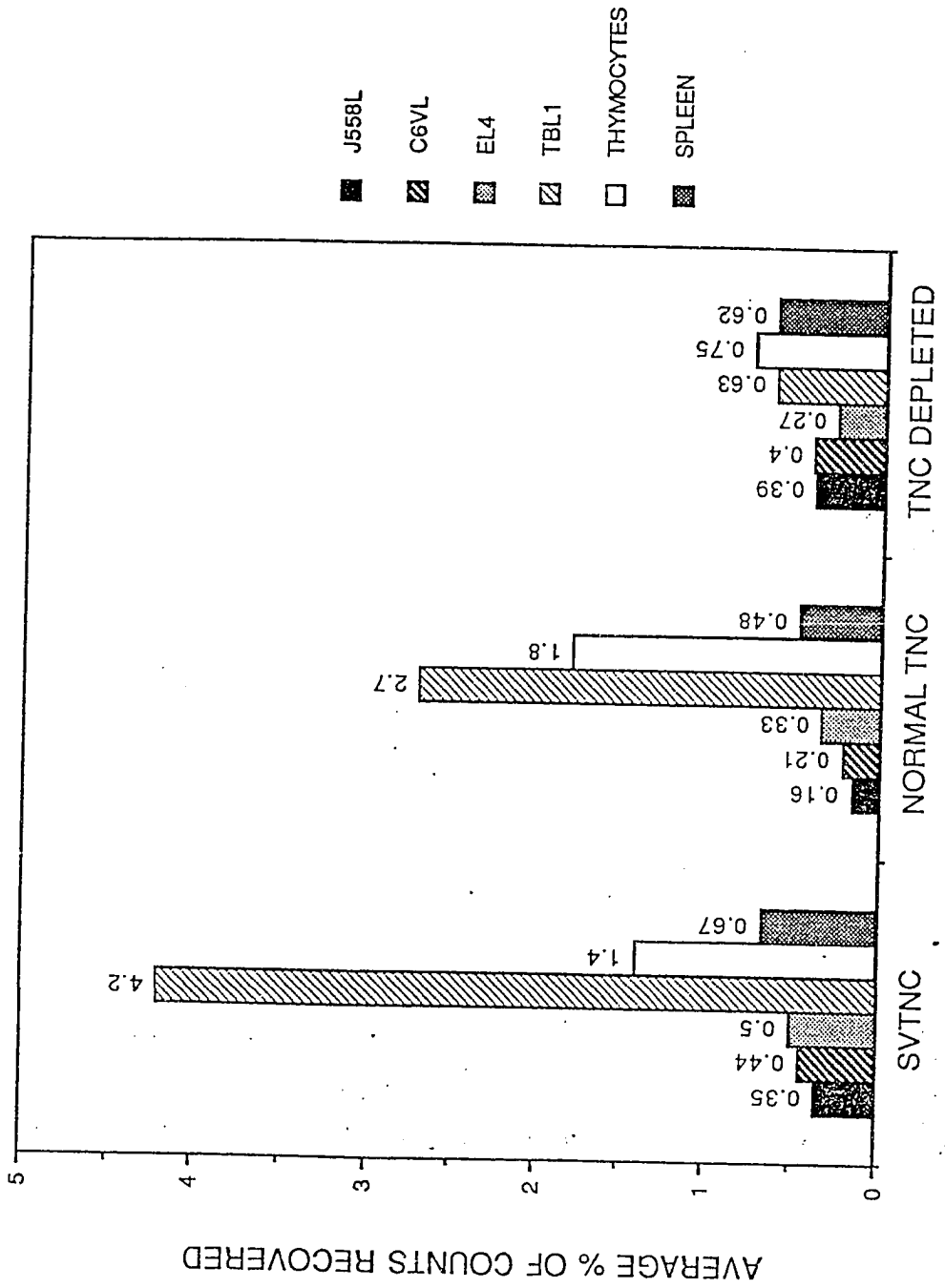


Figure 12. Internalization of bound lymphocytes by SVT-MP5. (A) Co-cultivation of TBLs and SVT-Mp5 cells after a 5 hour incubation in hanging drop cultures. Magnification 400 X. Both cell types were added simultaneously in 10 ml of growth media to a Terasaki well, and inverted immediately. (B) SVT-Mp5 cells were allowed to form a monolayer before the addition of TBL-1 cells under normal culture conditions. The photograph was taken after a 24 hour incubation period. Magnification 100 X. (C) Scanning electron micrograph of TBL-1 cells (marked A and B) bound to a SVT-Mp5 cell. (D) Several photographs of TBL-1 cells internalized by SVT-Mp5 cells. Vacuoles are visible around some of the cells. (E) The internalization of TBLs by a SVT-Mp5 nurse cell after co-cultivation overnight. Magnification 400 X. It is impossible to determine whether the thymocytes are inside or tightly bound to the top of the nurse cell at this magnification. (F) Scanning electron micrograph of the same cell shown in figure E. The arrow shows the TNC membrane above the lymphocytes. (G) A higher magnification of the same cell shown in figures E and F, showing the TNC membrane covering the TBL-1 cells.

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