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AND THEIR FUNCTION IN TUMOR DESTRUCTION.

The Ohio State University, Ph.D., 1974
Anatomy

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IN VITRO STUDIES ON THYMUS DERIVED LYMPHOCYTES:
DIFFERENTIATION OF T-LYMPHOCYTES AND THEIR FUNCTION IN
TUMOR DESTRUCTION

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by
Samuel David Waksal, B.A.

The Ohio State University
1974

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ACKNOWLEDGMENTS

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"What we call the beginning is often the end and to make an end is to make a beginning . . . . . We shall not cease from exploration and the end of all our exploration will be to arrive where we started and know the place for the first time".

- T. S. Eliot

For this reason (the Karaites) say to their brethren the children of Jacob - "Study, and search and seek, and investigate and do that which occurs to you by way of solid proof and that which seems reasonable to you".

- Sahl ben Masliah, the Karaite (from the Epistle to Jacob ben Samuel)
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GENERAL INTRODUCTION

Development of lymphocytes and their subsequent function in homograft and tumor rejection is of prime importance in the process of maintaining the integrity of the mammalian organism (1). These lymphocytes have been divided into two major populations, thymus-derived lymphocytes (T-lymphocytes) and bone marrow derived lymphocytes (B-lymphocytes) [2]. T-lymphocytes are involved in such cell mediated reactions as graft versus host reactivity (GVH), tumor cell destruction, and helper activity during antibody responses (3-5).

The presence of an intact thymus (6-8) is essential for the development of T-lymphocytes. Precursors of T-lymphocytes migrate to the thymus from either yolk sac or fetal liver during embryogenesis, and from bone marrow during adult life (9-10). The microenvironment of the thymus is responsible for T-lymphocyte differentiation and development either by the contact between precursors of T-lymphocytes and the thymus epithelium or through a hormonal influence (11). Thymic humoral factor, or thymosin, is putatively secreted by thymus epithelial cells (12) and is able to reconstitute T-lymphocyte function in thymectomized animals (13,14).
Thymocytes develop within the thymic microenvironment, and autoradiographic studies show that they migrate from cortical areas to medullary areas (15). This maturation process is characterized by changes in surface (differentiation) antigens as well as functional properties (16, 17). One of the more important surface antigens is the theta (θ) antigen, discovered by Reif and Allen, on mouse thymocytes (18) and shown by Raff to also exist on peripheral T-lymphocytes (19). This antigen has recently been termed Thy-1 (20). Less differentiated thymocytes contain large amounts of Thy-1, TL (thymus leukemia) antigens, and small amounts of H-2 antigen on their surfaces (21). The more differentiated thymocytes contain large amounts of H-2, small amounts of Thy-1, and no TL antigen (21). The highly differentiated thymocytes in the medullary regions of the thymus acquire GVH reactivity and cortisone resistance (22).

Peripheral T-lymphocyte populations are derived from cells which migrate from the thymus. This has been shown in studies using in situ labeled thymocytes, chromosomally labeled thymus grafts, and depletion of T-lymphocyte numbers in peripheral lymphoid organs after neonatal thymectomy (23-26). The studies on neonatal thymectomy showed the depletion of certain areas in peripheral lymphoid organs, which were termed by Parrot, et al. as thymic-dependent areas (27). These were the periarteriolar areas of the
spleen and deep cortical areas of lymph nodes. The demonstration of Thy-1 antigen on peripheral T-lymphocytes further substantiated the above studies (19, 26).

Studies by Cantor and Asofsky (28), using GvH in vivo and Waksal, et al. (29) in vitro, indicates that peripheral T-lymphocytes are also a heterogeneous cell population. These studies have suggested that less differentiated T-lymphocytes are located primarily in the spleen, while more differentiated T-lymphocytes are located in lymph nodes, thoracic duct, and peripheral blood. The less differentiated T-lymphocytes (T1) do not recirculate, have large amounts of Thy-1 antigen on their surface membranes, and are dependent on an intact thymus for their existence. Highly differentiated T-lymphocytes (T2) are rapidly recirculating cells, contain small amounts of Thy-1 antigen, and are not dependent on the existence of a thymus.

Less differentiated T-lymphocytes are precursors of those cells which are reactive in the GVH, while the highly differentiated T-lymphocytes are the amplifier cells in the GVH (30). Segal, et al. have shown that T-lymphocyte populations can be distinguished functionally by hydrocortisone (31). Cortisone sensitive T-lymphocytes take part in carrier recognition during antibody responses to T-dependent antigens while cortisone resistant cells induce GVH.
T-lymphocytes in various stages of development or separate T-lymphocyte subpopulations may play an important role in immune surveillance against neoplastic cells (34). Many in vitro techniques have been developed to study the development of effector lymphocytes in vitro against transplantation or tumor antigens (35-37). These studies, as well as their in vivo correlates, suggest that cell-mediated immune reactions can be divided into three phases. An afferent limb or recognition phase where immunogens are recognized by specific receptors on T-lymphocytes, a central phase of lymphocyte proliferation, and the efferent limb or effector phase in which cytotoxic lymphocytes destroy specific target cells (38, 39).

The sensitization phase has been accomplished in both human and animal systems using two separate approaches. In one method, lymphocytes have been sensitized in vitro using a modified one-way mixed lymphocyte reaction (MLR) [35, 40, 41]. The cells sensitized by an MLR were capable of destroying allogeneic target cells of the same antigenic type as the sensitizing cells. Using this method murine lymphocytes have also been sensitized against syngeneic plasma cell tumors (42). The second method uses fibroblast monolayers as stimulating cells. Using this method, rat or mouse lymphocytes underwent blast transformation and stimulation in culture (43). The cells generated in this manner were subsequently able to effect cytolysis of target cells antigenically identical to the sensitizing monolayers. Using antisera directed against surface
markers on lymphocytes, these particular reactions were shown to be mediated by T-lymphocytes rather than B or K lymphocytes which take part in antibody mediated target cell lysis (37, 40).

Parts I and II of this dissertation are directed toward elucidating the initial induction process of T-lymphocyte development as well as the differentiation or control processes governing the function of peripheral T-lymphocyte subpopulations. Part III is directed toward adaptation of the in vitro methods, discussed above, to study T-lymphocyte activity during culture on virus transformed syngeneic tumor monolayers.
PART I

INDUCTION OF T-LYMPHOCYTE DIFFERENTIATION

IN VITRO BY THYMUS RETICULOEPITHELIAL CELLS

INTRODUCTION

The development of thymus-derived lymphocytes (T-lymphocytes) is dependent upon the existence of an intact thymus (6-8). T-lymphocytes are responsible for mediating such immunological activities as homograft rejection, graft-versus-host reactivity (GVH), and regulation of antibody production to certain antigens by B-lymphocytes (1, 3, 5). T-lymphocytes are a heterogeneous cell population (32). The existence of T-lymphocyte subpopulations may be an expression of maturational changes which occur during T-lymphocyte development. T-lymphocytes are also characterized by the presence of surface antigens, such as Thy-1 in mice, and by their characteristic responses to mitogens such as phytohemagglutinin (PHA) and concanavalin A (Con A) [44, 45].

The thymus develops from the epithelial anlage of the 3rd and 4th pharyngeal pouches and becomes a continuous epithelium of cells infiltrated by developing thymocytes (46, 47). Precursors to T-lymphocytes from the yolk sac or the fetal liver during embryogenesis and from the bone marrow during adult life migrate to the
thymus where they differentiate into thymocytes under the influence of the thymic microenvironment (9, 10). The thymus produces a humoral factor which may control some aspects of T-lymphocyte differentiation (48). This humoral factor is putatively secreted by the reticuloepithelial cells of the thymus (TE cells) [11]. Thymic factors have been isolated from the thymuses of many mammalian species. These thymic factors can cross species barriers and effect changes in T-lymphocyte development (13).

It is not known whether contact between precursor cells of T-lymphocytes and TE cells is necessary for the initial induction of T-lymphocyte development or whether the thymus acts through a purely humoral mechanism.

The present study attempts to elucidate the role of TE cells in the differentiation process of T-lymphocytes. The TE cells were grown in culture devoid of any lymphocytic elements, thus making it possible to examine the ability of these cultures to induce T-lymphocyte development while ruling out thymocyte influence.
MATERIALS AND METHODS

Animals: 4-6 week old female C57Bl/6 BDF₁ (C57Bl/6X DBA/2)F₁ mice and female Lewis (Le) rats obtained from Jackson Laboratories, Bar Harbor, Maine, are maintained on Purina laboratory chow and water ad libidum. Congenitally athymic mice ("nude") containing the nu/nu locus and congeneric with C57Bl/6 and obtained from Dr. David Osoba, Toronto, Canada are bred in aseptic conditions at the Graves Hall Vivarium.

T-lymphocyte deficient mice: Six week old mice are thymectomized by a modification of the method of Miller (49). Mice are anesthetized with ether, the sternum is split and the thymus is removed with small surgical forceps. Two weeks after thymectomy, the mice are irradiated with 900R for 14.7 minutes using a Cesium 137 source. Immediately after irradiation the mice are injected with 5x10⁶ syngeneic bone marrow cells that have been treated with anti-Thy-1 serum and guinea pig complement. Six weeks after bone marrow transplant, T-lymphocyte deficient mice are used as the source of precursor cells. At
sacrifice, only mice showing no thymic remnants were used.

Supporting cell monolayers: Thymus-epithelial monolayers are prepared by aseptically removing thymuses from C57Bl/6 mice and Lewis rats and mincing the thymuses until all solid pieces are removed. The minced thymus tissue is put into a trypsinizing flask. The tissue is washed in Hank's balanced salt solution (HBSS) for 10 minutes using a magnetic stirrer. The HBSS is then poured off and replaced with 0.25 percent trypsin-EDTA solution (Gibco) and trypsinized for 10 minutes. The tissue is put into a flask containing 0.05 percent collagenase solution (Type III, Sigma Chemical Co.) and allowed to digest overnight at 4°C. The fragments which are swollen and partially dispersed are further dispersed by vigorous pipeting and poured through double pieces of sterile gauze to remove remaining solid tissue pieces. The cells are centrifuged at 800G and washed in cold HBSS. The cells are then added to falcon plastic 60 mm tissue culture dishes with Waymouths medium without serum. Two mouse thymuses per plate and one rat thymus per two plates are used as the concentration for culturing the cells. The Waymouths medium does not allow for lymphocyte survival but does allow for growth of the adherent cell population. After two days of culture, the falcon dishes are washed with cold HBSS, Eagle's
minimal essential media (MEM, Gibco), + 15 percent fetal calf serum (FCS) is added and the cells maintained as a continuous line. Secondary cell cultures are prepared by trypsinizing the primary cell cultures with 0.1 percent trypsin. Only secondary cell monolayers are used in the experiments to rule out the possibility of contaminating thymocytes.

Fibroblast monolayers are prepared by mincing 14-16 day old mouse embryos and trypsinizing them for 10 and 20 minutes in 0.25 percent trypsin-EDTA solution. The cells are maintained on MEM + 10 percent FCS.

Cell cultures: Spleens are removed aseptically from adult thymectomized irradiated bone marrow reconstituted (ATxBM) mice or "nude" mice and teased in cold, sterile HBSS to obtain a single cell suspension. The cells were collected by centrifugation at 180G for 10 minutes at 4°C and resuspended in MEM + 10 percent FCS at a concentration of 1x10^7 cells per ml. Bone marrow cells from ATxBM mice are obtained by removing femurs and flushing out the bone marrow with MEM + 10 percent FCS using an 18 gauge needle. The bone marrow cells are washed and resuspended at a concentration of 1x10^7 cells per ml. Three ml of spleen or bone marrow cells are added to TE monolayers or control fibroblast monolayers and incubated for 24 hours. The cells are removed from the monolayers by gentle pipetting and examined for acquisition of T-lymphocyte characteristics.
Mitogen stimulation: One ug per ml final concentration of Con A (Miles Yeda Laboratories) was used for stimulation. Cells were cultured at a density of 1x10^6 per ml in 1 ml volumes. DNA synthesis was assayed using tritiated methyl thymidine (HdTR) at 72 hours. Twenty-four hours prior to harvest 0.5 mCi HdTR (ICN) is added to each culture tube. Cultures were washed twice with cold HBSS, twice with cold 5 percent trichloracetic acid and once with cold 95 percent ethanol. The precipitates were dissolved in 15 ml of "Aquasol" liquid and placed in plastic screw-cap tubes and counted in a Packard scintillation spectrometer. Triplicate cultures were performed for each experimental group.

Graft-Versus-Host response: Cells removed from the thymus-epithelial cultures or from the control fibroblast cultures are suspended at a concentration of 10^7 cells in a volume of 0.05 ml HBSS and injected into the right footpads of irradiated (900R) BDF₁ recipient mice, using a syringe with a 27 gauge needle. The F₁ hybrids were divided into 2 groups containing 6 mice per group. One group had cells cultured on TE monolayers injected in the right footpad with HBSS injected into the left footpad. The second group had cells from control monolayers injected into the right footpad with HBSS injected into the left footpad. The right and left popliteal lymph nodes are removed after six days and weighed. The lymph node index is computed as a mean ratio of the right to
left lymph node weight. Statistical significance of the difference between experimental groups and control groups is measured using the Student t-test.

**Preparation of mouse brain antiserum:** Anti-mouse-brain-serum is prepared using a modification of the method of Golub (50). Brains from 5 adult male C3H/Hej mice were homogenized in HBSS. The homogenate is adjusted to a volume of 2.5 ml with HBSS and added to an equal volume of Freund's complete adjuvant. Rabbits were injected subdermally at six sites with 1.0 ml of the homogenate. The rabbits were bled 14 days after the second of two weekly injections. The antiserum was pooled and, after absorption, stored at -20°C.

**Quantitation of Thy-1-bearing lymphocytes:** The number of cultured precursor cells bearing the Thy-1 antigen was determined by a modification of the technique of Gorer and O'Gorman (51). Single cell suspensions were prepared from thymus, spleen, and lymph nodes. The concentration was adjusted to 5x10⁶ viable cells/ml, as determined by trypan blue exclusion. Five hundredths ml quantities of antisera, lymphocytes (5x10⁶/ml), and guinea pig complement (diluted 1:4) were placed in tubes and incubated for 45 minutes at 37°C in 5 percent CO₂. Viability was determined by trypan blue exclusion.
RESULTS

**Thymus reticuloeipithelial cell cultures:** After eight hours of incubation adherent cells appeared in the cell cultures. After ten days of culture the majority of the lymphocytes were removed and by day 14 the cultures consisted of confluent monolayers. The monolayers contained mostly epithelial-like cells, and a few fibroblasts. The epithelial cells contain a large nucleus with two or more large nucleoli. The cytoplasm of many of the epithelial cells contained numerous refractile granules. The confluent monolayers did not contain any lymphocytic elements (Figs. 1, 2).

**Induction of mitogen responsiveness in T-lymphocyte deficient mice:** Single cell suspensions of spleen cells derived from normal or T-lymphocyte deficient C57Bl/6 mice were cultured on syngeneic TE monolayers or control fibroblasts for a period of 48 hours. The cells were collected and assessed for their ability to respond to Con A.

Table 1 shows the Con A responses of these cell populations as measured by uptake of H3 thymidine. The results are expressed as the ratio of the Con A treated group divided by the control group. The normal spleen cells show Con A ratios of 2.58, whereas
cells from T-deficient mice show a greatly diminished Con A response (ratio 0.87). Spleens from "nude" mice used as a source of precursor cells also showed induction of Con A responsiveness. Table 1 shows that cells from "nude" mouse spleen cultured on control monolayers showed no increase in Con A responsiveness (ratio 1.2) whereas those cells cultured on TE monolayers had a Con A ratio of 3.5.

Table 2 shows that the TE monolayers were also able to induce Con A responses in bone marrow cells from T-lymphocyte deficient mice. Bone marrow cells from normal C57B1/6 mice had a Con A ratio of only 0.34. In contrast to the fibroblast control monolayers, bone marrow cells from ATxBM mice cultured on TE cells had a Con A ratio of 2.3.

**Comparative effects of syngeneic and xenogeneic TE monolayers:** A comparison was made of the effects of xenogeneic Le rat TE monolayers to those produced by syngeneic mouse TE cells. Table 3 shows that the xenogeneic TE monolayers give a stimulation index of 2.2 while those cells cultured on syngeneic TE monolayers give a stimulation index of 2.3. Therefore, the xenogeneic TE monolayers are as efficient as syngeneic TE monolayers in the induction of Con A responsiveness.

**Induction of graft-versus-host reactivity:** Table 4 shows that normal spleen cells cultured on either syngeneic TE or control
monolayers give GVH responses of 5.4 and 5.9 (lymph node index). Cells from T-deficient mice cultured on fibroblast monolayers show GVH reactivity of only 2.1 lymph node index, whereas cells cultured on TE monolayers give a GVH response of the same magnitude as normal spleen lymphocytes (5.9).

_Acquisition of Thy-1 antigen:_ Spleen cells from normal or T-lymphocyte deficient mice were cultured on either syngeneic TE monolayers or control fibroblast monolayers for 48 hours. Anti-mouse brain sera shown to be specific for mouse T-lymphocytes was used in a concentration of 1:8 in a cytotoxicity assay. Normal spleen cells cultured on either the TE or fibroblast monolayers showed 32 and 37 percent cytotoxicity respectively while cells from T-lymphocyte deficient mice cultured on fibroblast monolayers showed less than 5 percent cytotoxicity. After culture on TE monolayers spleen cells from these mice showed 27 percent cytotoxicity (Table 5).
DISCUSSION

This work suggests that the thymus reticuloepithelial cells play an important role in the inductive processes involved with T-lymphocyte differentiation.

It is well known that the thymic microenvironment is essential for the development of T-lymphocytes. Neonatally thymectomized and mice which are congenitally athymic lack the development of T-lymphocyte dependent areas in the peripheral lymphoid organs. Precursors of T-lymphocytes migrate from the yolk sac and liver during embryogenesis and from the bone marrow during adult life, to the thymus where development and maturation occurs (9, 10). Developing thymocytes migrate through the cortex of the thymus where immature thymocytes reside to the medullary regions where more highly differentiated cells reside (15). Clark has shown that the cortical epithelial cells differ morphologically from those epithelial cells in the medullary areas. These various types of epithelial cells may play a role in the induction of differentiation of the various subpopulations of T-lymphocytes (11). Autoradiographic
studies suggested that the cortical epithelial cells of the thymus were secretory in nature and may be responsible for the production and secretion of thymic hormone (12).

Thymocyte maturation is characterized by changes in surface antigens such as Thy-1 acquisition in the mouse (16), acquisition of reactivity to Con A and PHA (22) and acquisition of the ability to mount a graft-versus-host reaction (3). Precursor cells from T-lymphocyte deficient mice cultured on TE cells in the present study developed the ability to respond to Con A as well as the ability to mount a GVH. Culturing precursors of T-lymphocytes on TE monolayers also induced the expression of Thy-1 antigen.

The mechanism by which the microenvironment of the thymus induces T-lymphocyte differentiation is not well understood. The process may involve secretion of thymic hormone by the thymus epithelium and interaction between the hormone and the precursor cells, or cell-to-cell interaction between the epithelium and the precursor cells. The thymic microenvironment may affect not only T-lymphocyte differentiation but may also influence thymocytes during their various maturation changes.

This study shows that the reticular epithelial cells of the thymus may be the cells specifically involved in the initial
induction of T-lymphocyte differentiation. It also introduces a system to examine the events that take place during T-lymphocyte maturation.
SUMMARY

Monolayers of thymus reticular epithelial cells were cultured devoid of any lymphocytic elements. These cultures contained secretory cells which were able to induce responsiveness to Concanavalin A, graft versus host reactivity, and acquisition of Thy 1 antigen in precursor cell populations from bone marrow and spleens of adult thymectomized irradiated and bone marrow reconstituted mice and spleens from congenitally athymic "nude" mice. It was also shown that xenogeneic rat thymuses reticular epithelial monolayers were as effective in the induction of T-lymphocyte differentiation in vitro as syngeneic thymus reticular epithelial monolayers. This system is therefore ideal for the study of T-lymphocyte development.
<table>
<thead>
<tr>
<th>MOUSE SPLEEN CELLS</th>
<th>SUPPORTING SYNGENEIC CELL MONOLAYERS</th>
<th>MEAN H(^3) THYMIDINE UPTAKE(^c)</th>
<th>RATIO-EXP/CONTROL(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL C57B1/6</td>
<td>FIBROBLASTS</td>
<td>8127 ± 836</td>
<td>3147 ± 421</td>
</tr>
<tr>
<td>ATxBM C57B1/6</td>
<td>FIBROBLASTS</td>
<td>1427 ± 123</td>
<td>1623 ± 89</td>
</tr>
<tr>
<td>ATxBM C57B1/6</td>
<td>TE CELLS</td>
<td>3142 ± 192</td>
<td>1561 ± 213</td>
</tr>
<tr>
<td>NU/NU</td>
<td>FIBROBLASTS</td>
<td>1651 ± 321</td>
<td>1421 ± 251</td>
</tr>
<tr>
<td>NU/NU</td>
<td>TE CELLS</td>
<td>6413 ± 422</td>
<td>1827 ± 188</td>
</tr>
</tbody>
</table>

\(^a\) Spleens from normal C57B1/6 mice and C57B1/6 mice that were thymectomized, lethally irradiated, and bone marrow protected (ATxBM) or spleens from nu/nu mice were cultured on syngeneic thymic epithelial (TE) cells or control fibroblast monolayers. TE cells were able to reconstitute the Con A response of T-deficient mice.

\(^b\) Con A ratios were computed by dividing the Con A treated CPM by the control CPM.

\(^c\) All tests were run in triplicate and the mean counts are pooled from 4 different experiments.
TABLE 2
EFFECT OF THYMUS EPITHELIUM ON CON A RESPONSES OF BONE MARROW CELLS

<table>
<thead>
<tr>
<th>MOUSE BONE MARROW CELLS</th>
<th>SUPPORTING SYNGENEIC CELL POPULATION</th>
<th>MEAN $^{3}H$ THYMIDINE UPTAKE$^c$</th>
<th>RATIO OF CON A/CPM/CONTROL$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C57Bl/6</td>
<td>FIBROBLASTS</td>
<td>4857 ± 197</td>
<td>2429 ± 289</td>
</tr>
<tr>
<td>ATxBM C57Bl/6</td>
<td>FIBROBLASTS</td>
<td>1256 ± 89</td>
<td>1487 ± 129</td>
</tr>
<tr>
<td>ATxBM C57Bl/6</td>
<td>TE CELLS</td>
<td>3565 ± 251</td>
<td>1521 ± 210</td>
</tr>
</tbody>
</table>

$^a$Bone marrow from normal C57Bl/6 mice and C57Bl/6 mice that were thymectomized, lethally irradiated, and bone marrow protected (ATxBM) were cultured on syngeneic thymic epithelial (TE) cells or control fibroblast monolayers. TE cells were able to reconstitute the Con A response of T-deficient mice.

$^b$Con A ratios were computed by dividing the Con A treated CPM by the control CPM.

$^c$All tests were run in triplicate and the mean counts are pooled from 4 different experiments.
<table>
<thead>
<tr>
<th>Spleen Cell Reactivity</th>
<th>Rat Fibroblasts (Le)</th>
<th>Rat TE Cells (Le)</th>
<th>Stimulation Index&lt;sup&gt;b&lt;/sup&gt;</th>
<th>C57B1/6 Fibroblasts</th>
<th>C57B1/6 TE Cells</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&lt;sup&gt;3&lt;/sup&gt; Thymidine Uptake in CPM (Mean)</td>
<td>1278 ± 291</td>
<td>2877 ± 628</td>
<td>2.2</td>
<td>1361 ± 213</td>
<td>3142 ± 192</td>
<td>2.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Shows the effects of culturing spleen cells from T-deficient C57B1/6 mice (ATxBM) on either xenogeneic Lewis (Le) rat thymic epithelial (TE) monolayers or syngeneic thymic epithelial monolayers. Results are expressed in the uptake of H<sup>3</sup> thymidine in counts per minute.

<sup>b</sup>Stimulation index is the ratio of the CPM of H<sup>3</sup> thymidine uptake by the cell cultures on the TE monolayers as compared to the control fibroblast monolayers.

<sup>c</sup>All tests were run in triplicate and the mean counts are pooled from 4 different experiments.
<table>
<thead>
<tr>
<th>CELL SOURCE MOUSE SPLEEN CELLS</th>
<th>SUPPORTING SYNGENEIC CELL MONOLAYERS</th>
<th>LYMPH NODE INDEX–POPLITEAL NODE ASSAY IN BDF₁ MICE&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C57Bl/6</td>
<td>FIBROBLASTS</td>
<td>5.4 ± 1.2 †</td>
</tr>
<tr>
<td>Normal C57Bl/6</td>
<td>TE CELLS</td>
<td>5.9 ± 1.4</td>
</tr>
<tr>
<td>ATxBM C57Bl/6</td>
<td>FIBROBLASTS</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td>ATxBM C57Bl/6</td>
<td>TE CELLS</td>
<td>5.9 ± 1.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Spleen cells from normal and T-deficient mice were cultured on thymus epithelial cells for 48 hours and injected (10⁷ cells/0.05 ml HBSS) into the right footpad of irradiated BDF₁ mice (C57Bl/6 x DVA/2 F₁ hybrids) to examine GVH response.

<sup>b</sup>Lymph node index was computed by comparing the lymph node weight of the right footpad to the left footpad.

†These ratios are computed from 3 separate experiments containing groups of 6 mice in each experiment.
### TABLE 5

**EFFECT OF THYMUS EPITHELIUM ON THY-1 (θ) REACTIVITY OF SPLEEN CELLS OF T-LYMPHOCYTE DEFICIENT MICE**

<table>
<thead>
<tr>
<th>MOUSE SPLEEN CELLSA</th>
<th>SUPPORTING SYNGENEIC CELL MONOLAYERS</th>
<th>% CYTOTOXICITY, ANTI-BRAIN SERUMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C57B1/6</td>
<td>FIBROBLASTS</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>Normal C57B1/6</td>
<td>TE CELLS</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>ATxBM C57B1/6</td>
<td>FIBROBLASTS</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>ATxBM C57B1/6</td>
<td>TE CELLS</td>
<td>27 ± 3</td>
</tr>
</tbody>
</table>

*The spleen cells were treated with anti-brain serum and guinea pig complement after a 24 hour incubation on either the fibroblast or thymus epithelial (TE) monolayers.*

*Anti-brain serum was used at a 1:8 dilution and guinea pig complement was used at a 1:4 dilution. Trypan blue uptake was used as the measure of cell death.*
PART II
FUNCTIONAL ACTIVITY OF SUBPOPULATIONS OF
THYMUS-DERIVED LYMPHOCYTES

INTRODUCTION

The discovery that two classes of lymphocytes exist has helped elucidate mechanisms of the immune response. One class, which differentiates in the bone marrow or the mammalian equivalent of the bursa of Fabricius, is termed B-cells (2). The second class is processed by the thymus and classified as T-lymphocytes (2). T-lymphocytes are a heterogeneous cell population which mediate such responses as homograft rejection, graft versus host reactivity (GVH), and helper function in antibody (3-5) responses. The subpopulations of T-lymphocytes have been characterized according to life span, organ localization, cortisone sensitivity, surface antigens, and functions (6-60). A less-differentiated T-cell population, recirculates slowly, contains high amounts of Thy-1 antigen and is located primarily in cortical areas of thymus and spleen (32). A more highly differentiated
T-cell population, recirculates rapidly, contains small amounts of Thy 1 antigen, and is located primarily in lymph nodes and thoracic duct (32).

Although recent studies have shown that adult thymectomy reduces the T-lymphocyte population of the spleen, and anti-thymocyte serum (ATS) primarily affects the lymph node T-lymphocytes, the nature and function of the T-lymphocyte populations which are affected by either adult thymectomy or anti-thymocyte serum is not presently known.
MATERIALS AND METHODS

**Mice:** 6-8 week old female BALB/C mice, C57Bl/6 female mice, and C3H/Hej mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. The mice were housed in plastic cages and fed Purina Laboratory Chow, with water ad libitum.

**Sensitizing Monolayers:** Tumor monolayers used for sensitization of lymphocytes were prepared by plating $2 \times 10^6$ tumor cells in 60 mm falcon plastic dishes. Target monolayers were prepared by adding $0.9 \times 10^6$ tumor cells to 35 mm falcon plastic dishes. Both sensitizing and target monolayers were irradiated with 2000R 24 hours after preparation. Target tumor monolayers were labelled with radioactive chromium by incubating them for 24 hours with Na$_2$CrO$_4$ (Amersham/Searle Radiochemicals), 2 μCi per target monolayer. The labelled target monolayers were incubated for another 48 hours and washed with Hanks balanced salt solution (HBSS) before using them in the cytotoxic assay.

**Sensitization of Lymphocytes in vitro on Tumor Monolayers:**
Lymph nodes (inguinal, axillary, brachial, cervical and mesenteric)
or spleens were removed from mice into cold PBS. Free cells were obtained by gently homogenizing the lymph nodes or spleens in a glass homogenizer with a loosely fitting piston and tissue fragments were allowed to sediment by gravity. The suspended lymphocytes were collected, centrifuged (600 x g, 7 min) and resuspended in Dulbecco's modified Eagle's medium (EM) containing 15 percent FCS or HS. Usually, $3.5 \times 10^6$ viable lymphocytes in 4 ml EM + HS were seeded on each sensitizing tumor monolayer. The cultures were incubated for 4 days in a humidified incubator with an inflow of 10 percent CO$_2$, in air. On the 3rd day, 3.5 ml of the culture medium was withdrawn from each plate and replaced by fresh culture medium. The sensitized lymphocytes were removed from the sensitizing tumor monolayers on the 4th or 5th day of culture by gentle pipeting of the monolayer surface.

**Sensitization in Mixed Lymphocyte Culture (MLR):** Single cell suspensions were made from thymus, spleen, and lymph nodes of C57Bl/6 mice and prepared by gentle homogenizing in a glass homogenizer (Balco glass); they were washed in balanced salt solution. Spleen cells from BALB/C mice were treated with Mitomycin C (40 µg/ml for 30 min at 37°C) in Eagle's medium and as the H-2d antigen source for the one-way MLR. Sixty million developing lymphocytes are cultured with $15 \times 10^6$ viable, Mitomycin C-treated spleen cells or leukemic cells in 3 ml of medium
in a glass tube sealed off by a dialysis membrane and suspended from the stopper of an Erlenmeyer flask containing tissue culture medium.

**Cytotoxicity Assay for MLR:** Sensitized lymphocytes obtained from MLR cultures are removed and washed with balanced salt solution. Viability counts are performed using trypan blue dye exclusion, and 5x10^6 lymphocytes mixed with 1x10^5 Cr^{51}-labelled tumor cells (in EM + 20% FCS) in culture tubes or in 35 mm Petri dishes on a rocker platform, and placed in a 10% CO_2, 90% air atmosphere at 37°C. Tumor cells are labelled with Cr^{51} by incubating 5x10^6 cells in 1 ml of EM + 20% FCS with 100 μCi of Cr^{51} for 30 minutes in 5% CO_2 at 37°C. Aliquots are taken from supernatants of experimental and control samples, counted in a Packard Gamma counter and percent cytotoxicity evaluated. All cytotoxicities are performed in triplicate cultures and the results are reported as the means of 4 separate experiments.

**Cytotoxicity Assay for Lymphocytes Sensitized on Monolayers:** The sensitized lymphocytes are centrifuged, resuspended in fresh EM + HS (or EM + FCS in some experiments) and the percentage of viable cells is determined. 3x10^6 sensitized lymphocytes in 1.5 ml culture medium are seeded on each target tumor monolayer. Cytotoxicity is assayed after 48 hours of incubation by measuring the amount of radioactivity released into the culture medium. Spontaneous release of Cr^{51} is
determined in every experiment by incubating target monolayers in medium without added lymphocytes. Cytotoxicity is expressed as the percent of Cr\textsuperscript{51} label released in experimental cultures, minus the spontaneous release. Cytotoxicities are performed in triplicate and the results reported are the means ± the standard deviation of 5 experiments.

**In vitro Labelling of Lymphocytes for Migration Studies:** Lymphocyte suspensions are prepared from the thymuses, spleens and lymph nodes as described above, or removed from cultures of \textit{in vitro} sensitized cells. The cells are resuspended in Eagle's minimum essential medium with 20% fetal calf serum and the cell concentration adjusted to \(10^8\) viable, nucleated cells/ml. The cells are incubated for 1 hour with gentle agitation with 25-50 \(\mu\text{Ci} \text{ Cr}\textsuperscript{51}-\text{sodium chromate (Amersham-Searle, 4.85\(\mu\text{g/Ci/ml}/10^8\) cells/ml. Following incubation, the labelled lymphocytes are washed three times in balanced salt solution and resuspended in balanced salt solution for inoculation, and a viability count performed.

**Lymphocyte Migration Studies:** Groups of 6-8 week old mice (6 mice/group) are injected intravenously with \(10^7\), Cr\textsuperscript{51} labeled thymus, spleen, or lymph node cells or cells removed from cultures of allogeneic fibroblast monolayers. Twenty four hours later, the mice are sacrificed, the lymphoid organs removed, and the percentage of radioactivity localizing in these tissues is determined. The amount of radioactivity in individual organs is
measured (6 mice/sample) in a Nuclear Chicago deep well gamma counter, and whole body counts are performed. The results reported are the means ± standard deviation of 10 experiments.

**Adult Thymectomy:** 6 week old mice were thymectomized by a modification of the method of Miller (49). Mice were anesthetized with ether, the sternum was split and the thymus was removed with small surgical forceps. The animals were examined for thymic remnants by gross examination before use in the experiments.

**Quantitation of Thy-1 Bearing Lymphoid Cells:** The frequency of cultured precursor cells bearing the Thy-1 antigen was determined by a modification of the technique of Gorer and O'Gorman (51). Single cell suspensions were prepared from thymus, spleen, and lymph nodes. The concentration was adjusted to $5 \times 10^6$ viable cells/ml, as determined by trypan blue exclusion. Five hundredths milliliter quantities of antisera (diluted 1:8), lymphocytes ($5 \times 10^6$/ml) and guinea pig complement (diluted 1:4) were placed in tubes and incubated for 45 minutes at 37°C in 5% CO$_2$. Viability was determined by trypan blue exclusion.
RESULTS

Homing Properties of Lymphocyte Subpopulations: Lymphocytes from lymph nodes, spleens, thymuses, and thymuses from C57Bl/6 mice treated with cortisone acetate were examined for homing characteristics by labelling with Cr51 and measuring percent localization in the various lymphoid organs.

Thymocytes showed the greatest percent localization in spleen while only 1.8% of the thymocytes migrated to the lymph nodes. Only 0.15% of the thymocytes recirculated to the thymus (Table 1). Splenic lymphocytes also migrated primarily to the spleen but showed a significant increase in percent localization in lymph nodes (Table 1). Lymphocytes derived from lymph nodes showed the highest percent localization in lymph nodes (Table 3). Neither lymphocytes from lymph nodes or spleen recirculated to the thymus (less than 0.1%). Cortisone resistant thymocytes showed a significant increase in lymph node localization over normal thymocytes (Table 1).

Effects of Allosensitization In Vitro on Migration Patterns of Lymphocyte Subpopulations: Since it has been postulated that encounter with antigen induces changes in the
differentiation state of T-lymphocytes, migration of lymphocytes from various lymphoid compartments was assessed after stimulation by alloantigen in vitro.

Lymphocytes from thymus, spleen, and lymph nodes of C₅₇Bl/6 mice cultured on allogeneic fibroblast monolayers (H-2ᵇ) were harvested, labelled with Cr⁵¹ and injected intravenously into syngeneic recipients. Unsensitized lymphocytes were labelled and injected as control monolayers.

Thymocytes and splenic lymphocytes each showed a significant increase in percent localization in lymph nodes while lymph node lymphocytes exhibited only a slight increase in the lymph node seeking population (Table 2).

In Vitro Reactivity of Lymphocyte Subpopulations Derived from Different Lymphoid Anatomical Compartments: Lymphocytes from various lymphoid compartments of C₅₇Bl/6 mice were sensitized against H-2ᵇ alloantigens in vitro and assessed for subsequent cytolytic activity against H-2ᵇ target cells (C₃H/hej mammary tumor). Cytotoxicity was assessed by measuring the amount of Cr⁵¹ released from target cells minus the control targets and compared to freeze-thawed labelled cells which gave maximum Cr⁵¹ release.

Lymphocytes from lymph nodes showed the greatest amount of cytotoxicity as measured by Cr⁵¹ release, 45.5%. This was the same cytotoxic potential shown by cortisone-resistant thymocytes obtained from animals injected 48 hours earlier with
2.5 mg of cortisone acetate (45.8%). Thymocytes from untreated mice showed the lowest amount of cytotoxicity, 12.0%, with splenic lymphocytes showing intermediate cytolytic activity (22.3%), [Fig. 1].

Effects of Adult Thymectomy and Antilymphocyte Serum (ALS) on Thy 1 (Ø)-positive Cells in Peripheral Lymphoid Organs: Spleens and lymph nodes from adult thymectomized C57Bl/6 mice were removed at 2, 4, 6 week intervals and single cell suspensions made. Anti-mouse brain sera specific for mouse T-lymphocytes was used to examine the amount of Thy 1 positive cells in the experimental and control populations. Spleens from ATx animals showed a significant decrease in Thy 1 positive cells while lymph nodes showed only a slight decrease in the T-lymphocyte population (Table 3).

In contrast, animals receiving small doses of anti-lymphocyte sera (ALS), 0.1 ml subcutaneously, effected a significant decrease in the T-lymphocyte populations 2 days after administration while Thy 1 positive cells in the spleen showed only a small decrease (Table 3).

In Vitro Reactivity of Lymphocytes from Spleens and Lymph Nodes of ATx Mice: Single cell suspensions of lymphocytes obtained from spleens and lymph nodes of ATx and control C57Bl/6 mice were sensitized against H-2d alloantigens in vitro as
described earlier.

Splenic lymphocytes from ATx animals showed an increase in their cytotoxic potential after in vitro sensitization compared to control mice (Table 4). Purified T-cell populations showed a more dramatic increase in their cytolytic ability after in vitro sensitization against alloantigens.

Lymphocytes obtained from lymph nodes of ATx mice showed no significant difference in cytotoxicity after sensitization as compared to lymph node lymphocytes from control mice (Table 4). Removal of B-cells using anti-mouse IgG or nylon wool columns to enrich the T-cell population does not change the results as was seen in the splenic preparations.

Effect of ATS on the In Vitro Reactivity of Lymphocytes Derived from Mouse Spleen or Lymph Nodes: Lymphocytes derived from spleens and lymph nodes of C57Bl/6 mice receiving 0.1 ml ATS subcutaneously or from control animals were sensitized against alloantigens H-2^d in vitro.

Splenic lymphocytes (mixed T and B cell population) from ATS treated animals showed no significant differences in their cytolytic activity against P815 mastocytoma cells (H-2^d) when compared to control animals. Purified T-cells from spleens of ATS treated mice showed a decrease in their cytotoxic ability when compared to untreated animals (Table 5).

Lymph node cells from ATS treated animals showed a dramatic decrease in their ability to effect cytotoxicity against
alloantigens as compared with normal controls. Purification procedures on ATS treated lymph nodes did not yield enough Thy-1 positive cells for significant in vitro studies to be done (Table 5).
DISCUSSION

These studies provide further support for the existence of at least two populations of thymus-derived cells. T1 cells, located in the spleen, seem to be under direct thymic control, while T2 cells located in the deep cortical areas of lymph nodes, are relatively unaffected by thymectomy. Following thymectomy, T1 cells either migrate out of the spleen or die, and spleens from thymectomized animals do not appear to regain this T-cell population. The T1 cells may represent a distinct short-lived subpopulation of a separate line of differentiation. The recirculating T2 cell seems to be a long lived population, which has the ability to transform to "killer" and/or memory cells upon antigenic stimulation. Migration studies showed homing patterns which correlated to organ compartmentalization. In similar studies of T-cell homing patterns in animals with lymphoid leukemia, we have found that the different differentiation states of neoplastic T-cells have a profound effect on both homing and subsequently on the course and nature of the disease (61). These data suggest that T-cells probably arise from a stem cell and sequentially advance along a single line of differentiation.
Data using autoradiography have shown that the cortisone-resistant thymocytes in the medullary areas of the thymus are derived from the cortisone sensitive population located in the cortical areas (15). In examining the effects of anti-Thy 1-sera on these areas of the thymus, we have noted that the cortex is depleted while the medullary areas remain relatively unaffected (55). Administration of anti-β-sera also results in depletion of the T-dependent areas of the spleen, while the lymph nodes remain unaffected. The present results on the effects of adult thymectomy showed similar relationships; T-dependent areas in spleen were affected while T-dependent areas in lymph nodes showed no effect. These data indicate that the state of differentiation antigens located on the surface of T-lymphocytes are functionally related. Changes in differentiation antigens or membrane configuration may control the migration patterns of the different T-cell subpopulations to target organs.

We propose that the thymus serves not only to induce progenitor cells to differentiate along T-cell lines, but also as a storehouse for cells in different functional states of differentiation. The majority of cells in the thymus are T1 cells which are located in the thymic cortex. A small percentage of these cells undergo differentiation to T2 within the thymus and are located in the thymic medulla. T2 cells from thymus probably migrate to lymph nodes and become part of the long-lived recirculating pool. The majority of T1 cells in the thymus
migrate to the spleen. Although the regulatory mechanism of this migration is not presently understood, it may reflect feedback mechanisms which control the release of cells from the thymus. This study shows that T-lymphocytes (T1) in the spleen are under immediate thymic influence, but can differentiate to lymph node seeking cells following antigenic stimulation. These cells become part of the long-lived recirculating pool. The proposed scheme for this differentiation and migration is described in Figure 2.
### TABLE 1

**Migration of Cr$^{51}$ Labeled Cells from Various Lymphoid Pools**

The percent counts are reported as the means ± standard deviation of 5 experiments.

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>Spleen</th>
<th>Lymph Nodes</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>29.1 ± 3.6</td>
<td>0.18 ± 0.61</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>40.3 ± 5.2</td>
<td>6.60 ± 1.70</td>
<td>0.12 ± 0.09</td>
</tr>
<tr>
<td>Lymph Node</td>
<td>36.9 ± 4.4</td>
<td>10.20 ± 2.40</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>Cortisone Resistant(^b) Thymocytes</td>
<td>32.5 ± 3.8</td>
<td>4.30 ± 0.90</td>
<td>0.07 ± 0.04</td>
</tr>
</tbody>
</table>

\(^a\) Single cell suspensions were prepared from thymus, spleen, lymph nodes, and cortisone treated thymus of C57B1/6 mice and labelled with radioactive Cr$^{51}$ (50 μCi/10^8 cells). 10^7 labelled lymphocytes were injected intravenously into 3 groups of syngeneic recipients (6 mice/group). The percent counts are reported as the means ± standard deviation of 5 experiments.

\(^b\) 2.5 mg of cortisone acetate was injected intraperitoneally 24 hours prior to removal of thymus.
TABLE 2

LYMPH NODE SEEKING CELLS (LSC) AFTER ALLOSENSITIZATION IN VITRO<sup>a</sup>

<table>
<thead>
<tr>
<th>LYMPHOCYTE SOURCE</th>
<th>BEFORE SENSITIZATION</th>
<th>PERCENT LSC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AFTER SENSITIZATION</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>THYMUS</td>
<td>0.18 ± 0.61</td>
<td>4.1 ± 0.39</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>SPLEEN</td>
<td>6.60 ± 1.70</td>
<td>12.2 ± 1.90</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>LYMPH NODE</td>
<td>10.20 ± 2.40</td>
<td>14.3 ± 2.30</td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Single cell suspensions from thymus, spleen and lymph nodes of C57B1/6 mice were cultured with mitomycin C treated BALB/C spleen cells for 6 days.

<sup>b</sup>Groups of 6-8 week old C57B1/6 mice (6 mice per group) were injected intravenously with 10<sup>6</sup> Cr<sup>51</sup> labelled thymus, spleen, and lymph node cells from the allogeneic sensitization cultures. Lymphoid organs were removed 24 hours later and the percent radioactivity localized in the tissues was determined in a nuclear Chicago deep well Gamma counter. The results are reported as the means ± the standard deviation of 10 experiments.
TABLE 3

PERCENT THY-1 BEARING PERIPHERAL T-LYMPHOCYTES AFTER ADULT THYMECTOMY

<table>
<thead>
<tr>
<th></th>
<th>LYMPH NODE</th>
<th>SPLEEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C57Bl/6 mice</td>
<td>65.2 ± 3.7</td>
<td>33.7 ± 5.6</td>
</tr>
<tr>
<td>Adult Thymectomized C57Bl/6 mice</td>
<td>56.3 ± 2.1</td>
<td>12.4 ± 1.8</td>
</tr>
</tbody>
</table>

Lymph nodes and spleens were removed from adult thymectomized C57Bl/6 mice 2 weeks after thymectomies were performed and from normal C57Bl/6 mice, and single cell suspensions were prepared to examine the number of Thy-1 bearing lymphocytes. The concentration of the cell suspensions was adjusted to 5x10^6 viable cells/ml and treated with anti-mouse brain serum (diluted 1:8) plus guinea pig complement (diluted 1:4). Viability was determined by trypan blue exclusion. The results are reported as the means ± standard deviation of 4 experiments.
### TABLE 4

<table>
<thead>
<tr>
<th>PERCENT THY-1 BEARING PERIPHERAL T-LYMPHOCYTES AFTER TREATMENT WITH ANTI-THYMOCYTE SERUM(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Lymph Node</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>Normal C57Bl/6</td>
</tr>
<tr>
<td>ATS treated C57Bl/6 mice</td>
</tr>
</tbody>
</table>

\(^a\)0.1 ml of anti-thymocyte serum was injected into groups of 6 week old C57Bl/6 mice (6 mice per group) subcutaneously and lymph nodes and spleens were removed 3 days post-injection. Single cell suspensions were prepared to examine the number of Thy-1 bearing lymphocytes. The concentration of the cell suspensions was adjusted to 5x10^6 viable cells/ml and treated with anti-mouse brain serum (diluted 1:8) plus guinea pig complement (diluted 1:4). Viability was determined by trypan blue exclusion. The results are reported as the means ± standard deviation of 4 experiments.
TABLE 5
PERCENT CYTOTOXICITY OF IN VITRO SENSITIZED LYMPHOCYTES
FROM ADULT THYMECTOMIZED (ATx) MICE

<table>
<thead>
<tr>
<th></th>
<th>ATx^a</th>
<th>NORMAL CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node lymphocytes^b</td>
<td>46.7 ± 2.1^c</td>
<td>43.2 ± 4.5</td>
</tr>
<tr>
<td>Splenic lymphocytes</td>
<td>45.3 ± 0.8</td>
<td>27.4 ± 2.3</td>
</tr>
</tbody>
</table>

^aLymph nodes and spleens were removed from adult thymectomized C57Bl/6 mice 2 weeks after thymectomy was performed and from normal C57Bl/6 mice and single cell suspensions were prepared. The single cell suspensions of lymphocytes from adult thymectomized or normal C57Bl/6 mice were then cultured with mitomycin C treated BALB/C spleen cells for 6 days. The lymph nodes and spleens were removed from groups of 10 ATx mice.

^bSensitized lymphocytes obtained from the mixed lymphocyte reaction were mixed with Cr^51 labelled P-815Y mastocytoma cells at a ratio of 50:1 (5x10^6 lymphocytes and 1x10^5 Cr^51 labelled tumor cells)

^cAll cytotoxicities were performed in triplicate cultures and the results reported as the means ± standard deviation of 4 separate experiments.
### TABLE 6

PERCENT CYTOTOXICITY OF IN VITRO SENSITIZED CELLS FROM ANTI-THYMOCYTE SERUM (ATS) TREATED MICE

<table>
<thead>
<tr>
<th></th>
<th>ATS TREATED&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NORMAL CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node cells&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.4 ± 3.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.2 ± 4.5</td>
</tr>
<tr>
<td>Splenic lymphocytes</td>
<td>12.4 ± 2.7</td>
<td>27.4 ± 2.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>0.1 ml of anti-thymocyte serum was injected into groups of 6 week old C57BL/6 mice (6 mice/group) subcutaneously and lymph nodes and spleens were removed 3 days post-injection. Single cell suspensions of lymphocytes from ATS treated or normal mice were then cultured with mitomycin C treated BALB/C spleen cells for 6 days.

<sup>b</sup>Sensitized lymphocytes obtained from the mixed lymphocyte reaction were mixed with Cr<sup>51</sup> labelled P-815Y mastocytoma cells at a ratio of 50:1 (5x10<sup>6</sup> lymphocytes and 1x10<sup>5</sup> Cr<sup>51</sup> labelled tumor cells)

<sup>c</sup>All cytotoxicities were performed in triplicate cultures and the results reported as the means ± standard deviation in 4 separate experiments.
INTRODUCTION

The participation of the immune system in the elimination of neoplastic states is suggested by the higher incidence of tumors in patients with naturally occurring immune deficiency diseases (62), in autoimmune diseases (63), and in patients treated by immunosuppressive agents. These findings, coupled with experimental studies in animals, have led to the concept that immune surveillance mechanisms operate against potentially malignant cells in all multicellular organisms (1).

The host response during tumor rejection is a cell-mediated response directed against tumor associated antigens (TAA) which are not present on normal cells (65, 66).

The inability of the immune system to detect or destroy the small numbers of neoplastic cells which initiate primary or metastatic tumors is not completely understood. The mechanisms
may include insufficient immunogenicity (65), high or low dose tolerance (67), or blocking of receptors on immunocompetent lymphocytes by antigen-antibody complexes in the serum of tumor bearing patients (68).

Since the immune system is capable of overcoming only a limited number of tumor cells (66), immunotherapy may be useful if it could provide the patient with a sufficient population of lymphocytes already sensitized against TAA bearing neoplastic cells.

Lymphocytes cultured on xenogeneic or allogeneic fibroblasts became sensitized against surface antigen components present on the fibroblasts and were subsequently able to specifically lyse target cells syngeneic to the sensitizing fibroblasts (36, 37). These reactions were shown to be mediated by T-lymphocytes rather than B-lymphocytes or macrophages (37). Lymphocytes sensitized in vitro against alloantigens injected into syngeneic recipients were able to confer immunity against tumor cells bearing the same H-2 antigens as the sensitizing fibroblasts (69). The lymphocytes sensitized in vitro against alloantigens were also able to recruit a population of T-lymphocytes which prevented tumor growth in the host inoculated with tumor cells of the same H-2 phenotype (70). Therefore, the use of such culture systems by which specifically sensitized lymphocytes are obtained may also be an
effective means of obtaining lymphocytes sensitized against syngeneic TAA bearing tumor cells.
MATERIALS AND METHODS

Mice: 6-8 week old BALB/C mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. The mice were housed in plastic cages and fed Purina Laboratory Chow with water ad libitum.

Tumor Cell Monolayers: SV40 transformed BALB/C 3T3 cells (SVT2) and normal BALB/C 3T3 cells clone A31, subclone 7, obtained from Dr. M. Lieber of Meloy Laboratories were grown in Dulbecco's modified Eagle's medium supplemented with 10% Colorado calf serum. Cultures were maintained at 37°C in an atmosphere of 10% CO₂.

Sensitizing Monolayers: Tumor monolayers used for sensitization of lymphocytes were prepared by plating 2x10⁶ tumor cells in 60 mm falcon plastic dishes. Target monolayers were prepared by adding 0.9x10⁶ tumor cells to 35 mm falcon plastic dishes. Both sensitizing and target monolayers were irradiated with 2000R 24 hours after preparation in order to stop cell division during the sensitization process. Target tumor monolayers were labelled with radioactive chromium by incubating them for 24 hours with Na₂⁵¹CrO₄ (Amersham-Searle Radiochemicals), 2 μCi per target monolayer. The labelled target
monolayers were incubated for another 48 hours and washed with Hanks balanced salt solution (HBSS) before using them in the cytotoxic assay.

**Sensitization of lymphocytes in vitro:** Lymph nodes (inguinal, axillary, brachial, cervical and mesenteric) or spleens were removed from mice into cold phosphate buffered saline (PBS). Free lymphocytes were obtained by gently homogenizing the lymph nodes or spleens in a glass homogenizer with a loosely fitting piston and tissue fragments were allowed to sediment by gravity. The suspended lymphocytes were collected, centrifuged (600 x g, 7 min) and resuspended in Dulbecco's modified Eagle's medium (EM) containing 15% FCS or HS. Usually, 35x10^6 viable lymphocytes in 4 ml EM + HS were seeded on each sensitizing tumor monolayer. The cultures were incubated for 4 days in a humidified incubator with an inflow of 10% CO_2, in air. On the 3rd day, 3.5 ml of the culture medium was withdrawn from each plate and replaced by fresh culture medium. The sensitized lymphocytes were removed from the sensitizing tumor monolayers on the 4th or 5th day of culture by gentle pipetting of the monolayer surface.

**Cytotoxicity Assay:** The sensitized lymphocytes were centrifuged, resuspended in fresh Eagle's minimum essential medium and fetal calf serum (15%) and the percentage of viable cells determined. 3x10^6 sensitized lymphocytes in 1.5 ml
culture medium were seeded on each target tumor monolayer. Cytotoxicity was assayed after 42-46 hours of incubation by measuring the amount of radioactivity released into the culture medium. Spontaneous release of $^{51}$Cr was determined in every experiment by incubating target monolayers in medium without added lymphocytes. Cytotoxicity was expressed as the percent of $^{51}$Cr label released in experimental culture, minus the spontaneous release.

**Immunoadsorbent Monolayers:** Monolayers are used as immunoadsorbents by incubating 50-60x$10^6$ lymphocytes in 2 ml EM + FCS on each monolayer. At 1, 2, and 3 hours, 1 ml of EM + FCS was added to each culture, and the plates were rotated for 5 minutes at 50 rpm at room temperature on a mechanical rotator. The nonadherent lymphocytes were separated from those which adhered to the monolayer surface, by washing the monolayer surface with the culture medium. 80-95% of the lymphocytes originally seeded were usually recovered. The monolayers with the adhering lymphocytes were washed once with PBS to remove residual nonadhering cells, and 4 ml of EM + FCS was added to each culture. The nonadhering cells were counted and 35x$10^6$ lymphocytes in 4 ml EM + FCS were seeded on new sensitizing tumor monolayers as described earlier. Sensitization of the adherent and the nonadherent lymphocytes was carried out as described above, with unadsorbed
lymphocytes serving as controls. All sensitization was done in triplicate cultures.

**Recruitment:** Sensitized lymphocytes removed from tumor monolayers were collected and irradiated with 1000R. $1 \times 10^7$ irradiated cells were injected into the right footpad of syngeneic recipient mice while unsensitized control lymphocytes were injected into the left footpad. Regional lymph nodes (popliteal) were removed from the recipient animals 6 days later and single cell suspensions from experimental and control lymph nodes were used in the cytotoxicity assay. Groups of 6 animals for each experiment were injected with sensitized lymphocytes and control lymphocytes.

**Treatment with anti-mouse brain serum:** The lymphocytes sensitized against SVT2 monolayers were treated with anti-mouse brain serum prior to cytotoxicity tests. The concentration was adjusted to $5 \times 10^6$ viable cells/ml, as determined by trypan blue exclusion. Five hundredths milliliter quantities of antisera (diluted 1:8), lymphocytes (5x10^6/ml) and guinea pig complement (diluted 1:4) were placed in tubes and incubated for 45 minutes at 37°C in 5% CO₂. Viability was determined by trypan blue exclusion.

**Lymphocyte migration to tumor grafts:** Groups of 6-8 week old mice (10 mice per group) are injected intravenously with $10^7$, Cr$^{51}$ labelled lymphocytes removed from cultures of syngeneic tumor monolayers. Twenty four hours later the mice
were sacrificed and their legs amputated below the popliteal nodes and the amount of radioactivity measured in a nuclear Chicago deep well Gamma counter. Percent localization was determined by dividing the total radioactivity injected by the amount of radioactivity contained in the footpads of the mice. The results were expressed as the means plus or minus the standard deviation of 6 experiments.
RESULTS

The lymphocytes sensitized for five days on SVT2 monolayers showed specific cytotoxicity against SVT2 target cells (39%) as compared to control 4198V target cells (13%) which were transformed by a polyoma rather than SV40 virus. However, the sensitized lymphocytes were also 20% cytotoxic against the control target cells consisting of normal 3T3 fibroblasts (Table 1).

Table 2 shows the results of the experiments where lymphocytes were adsorbed on normal 3T3 fibroblast monolayers prior to sensitization. Table 2 shows that there was no reduction in the cytotoxic ability of lymphocytes after adsorption against SVT2 target cells. The lymphocytes which were removed from the adsorbing monolayers were 41% cytotoxic as compared with 39% against SVT2 target cells for the lymphocytes which were not adsorbed. On the other hand, the lymphocytes which had been adsorbed on normal fibroblasts were only 9.5% cytotoxic against the normal target cells as compared to 20% cytotoxicity shown by those lymphocytes which had not been adsorbed. This was a 27% reduction in cytotoxic potential against normal 3T3 target cells.
Anti-mouse brain serum (anti-MB) specific for mouse T-lymphocytes and guinea pig complement abolished the cytotoxic activity of the in vitro sensitized lymphocytes as compared with those lymphocytes treated with control rabbit serum. The sensitized lymphocytes treated with the anti-MB effected only 9.3% cytotoxicity against the SVT2 target cells and 7.5% cytotoxicity against the 3T3 target cells while the sensitized lymphocytes treated with the control rabbit serum effected 36% and 21% cytotoxicity against the SVT2 and 3T3 target cells respectively (Table 3).

The sensitized lymphocytes examined for in vivo activity by being labelled with Cr⁵¹ intravenously into syngeneic recipients bearing SVT2 tumor grafts in one footpad and control cells in the other footpad showed significantly greater homing to the SVT2 tumor graft than the control graft. The SVT2 cells injected into either the right or left footpads to rule out increased vascularization in either footpad showed consistent significant homing to the SVT2 tumor graft. The sensitized lymphocytes showed 1.8% localization in the SVT2 graft as compared to the control tumor graft. The unsensitized lymphocytes showed 1.3% localization in the SVT2 graft as compared with 1.15% localization in the control graft, not a significant difference in percent localization (Table 4).
The popliteal lymph nodes of syngeneic recipients injected with irradiated \textit{in vitro} sensitized lymphocytes in one footpad were removed and were 4-6 times larger than the popliteal node receiving no lymphocytes. The lymphocytes from these popliteal nodes were 38\% cytotoxic against SVT\textsubscript{2} target cells and 23\% cytotoxic against 3T3 target cells. The lymphocytes from the popliteal nodes of groups of mice receiving unsensitized lymphocytes as a control were only 10\% cytotoxic against SVT\textsubscript{2} target cells and 11\% cytotoxic against 3T3 target cells. This level of cytotoxicity was equivalent to that effected by unsensitized normal lymphocytes taken from untreated mice (Table 5).
DISCUSSION

This study describes the specific induction of T-lymphocyte mediated cytotoxicity in vitro against TAA present on syngeneic virus transformed cell lines.

The induction of autosensitization in vitro described by Cohen (70) also occurs during sensitization against syngeneic tumor monolayers. Using techniques which are able to remove immunoreactive lymphocytes (71, 72) those cells which were reactive with self antigens were adsorbed on normal fibroblast monolayers, allowing those lymphocytes not reactive against self antigens to become sensitized only to TAA on the tumor cells. This system provides an effective way to produce specifically cytotoxic lymphocytes directed against TAA on tumor cells.

The use of a specific anti T-lymphocyte serum showed that cytotoxic cell populations consisted of T-lymphocytes. This correlates with the earlier studies in allogeneic systems which also showed that the cytotoxic reaction was mediated by T-lymphocytes (37).

Earlier studies had shown that lymphocytes sensitized in vitro were able to recruit lymphocytes in vivo to effect
specific cell killing in vitro against allogeneic fibroblasts which were antigenically identical to the sensitizing cells (38). The present study showed that the in vitro sensitized cells were also capable of recruiting lymphocytes in normal syngeneic recipients to effect cytotoxicity against tumor cells bearing the same TAA as the sensitizing tumor monolayer.

This model suggests that the production of specifically sensitized lymphocytes capable of providing immune surveillance might best be accomplished by inducing sensitization of host lymphocytes in cell culture outside the body. Studies showed that when in vivo sensitized cells were used a 10 to 200 times greater excess of lymphoid cells was necessary to produce tumor inhibition as compared to in vitro sensitized cells (73, 74). Culture of tumor cells leads to the elution of antibodies and the exposure of TAA (75). This may allow for enhanced immuno- genicity of poorly antigenic tumor cells. The ability of sensitized lymphocytes to recruit noncommitted cells may also be useful in immunotherapy. Noncommitted lymphocytes in a tumor bearing patient could be recruited by lymphocytes sensitized in vitro against TAA.

In summary, mouse lymphocytes sensitized against syngeneic tumor cells bearing TAA effects tumor immunity in vitro and in vivo. This appears to be a promising model for restoring specific immune surveillance against cancer cells in tumor bearing patients.
GENERAL SUMMARY

This dissertation has studied the differentiation and function of thymus derived lymphocytes. The results of this investigation show:

(1) cultures of thymus reticular epithelium devoid of any lymphocytic elements can induce T-lymphocyte differentiation in vitro as expressed by responsiveness to concanavalin A, acquisition of Thy 1 (θ) antigen, and graft versus host reactivity.

(2) lymphocytes derived from thymus, cortisone treated thymus, spleen, and lymph nodes have characteristic homing patterns and cytotoxic activity against alloantigens. Lymph nodes and cortisone resistant thymocytes showed the greatest cytotoxicity, thymocytes the least, and splenic lymphocytes intermediate cytotoxicity.
sensitization against alloantigens increasing migration to peripheral lymph nodes by thymocytes and splenic lymphocytes.

anti-thymocyte serum effects a great depletion of Thy-1 positive cells in the lymph nodes and a slight decrease in Thy 1 positive cells in the spleen. Anti thymocyte serum abolishes cytotoxic activity of lymph node T-lymphocytes and significantly reduces the activity of splenic T-lymphocytes.

adult thymectomy depletes Thy-1 positive cells in spleens while leaving lymph nodes unaffected. Adult thymectomy increased the cytotoxic activity of splenic T-lymphocytes while lymph node T-lymphocytes were not affected.

T-lymphocytes from spleens and lymph nodes became sensitized against syngeneic SV-40 virus transformed fibroblast monolayers in vitro after 5 days of culture.

lymphocytes sensitized in vitro against tumor associated antigens were able to kill target cells antigenically identical to the sensitizing monolayers
as well as allogeneic and xenogeneic cells transformed by SV-40 virus. These lymphocytes were also able to migrate to tumor grafts and were able to recruit non-sensitized lymphocytes in syngeneic recipients to kill the tumor cells in vitro.
TABLE 1

CYTOTOXIC ACTIVITY OF BALB/C LYMPHOCYTES SENSITIZED IN VITRO

<table>
<thead>
<tr>
<th>Effector Cells</th>
<th>% Cr\textsuperscript{51} release from target cells\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVT2 cells</td>
</tr>
<tr>
<td>Lymphocytes sensitized against SVT2 cells</td>
<td>39.3 ± 2.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Cytotoxicity was determined by comparing the radioactivity released from the experimental cultures to that released by the freeze thawed cultures. Control cultures consisted of unsensitized lymphocytes or cultures containing only labeled target cells. These cultures consistently had 10-14% Cr\textsuperscript{51} release. All cytotoxicity experiments were done in triplicate cultures and the results represent the means ± standard deviation of 5 experiments.
TABLE 2

SENSITIZATION OF BALB/C LYMPHOCYTES AGAINST SVT2 CELLS AFTER ADSORPTION ON 3T3 MONOLAYERS

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>% Cr$^{51}$ release from target cells$^a$</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before adsorption</td>
<td>after adsorption</td>
</tr>
<tr>
<td>SVT2</td>
<td>39.3 ± 2.8</td>
<td>41.7 ± 2.6</td>
</tr>
<tr>
<td>3T3</td>
<td>20.2 ± 1.3</td>
<td>9.5 ± 1.7</td>
</tr>
</tbody>
</table>

$^a$Cytotoxicity was determined by comparing the radioactivity released from the experimental cultures to that released by the freeze thawed cultures. Control cultures consisted of unsensitized lymphocytes of cultures containing only labeled target cells. These cultures consistently had 10-15% Cr$^{51}$ release. All cytotoxicities were performed in triplicate cultures and the results represent the means ± standard deviation of 5 experiments.
TABLE 3

EFFECT OF ANTI-MOUSE BRAIN SERUM (anti Thy-1) ON ANTITUMOR ACTIVITY IN VITRO

<table>
<thead>
<tr>
<th>Sensitized lymphocytes (a)</th>
<th>%$^{51}$Cr Release from target cells b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated with anti mouse brain</td>
<td>9.3 ± 1.5</td>
</tr>
<tr>
<td>Treated with control serum</td>
<td>36.7 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>7.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>21.2 ± 2.8</td>
</tr>
</tbody>
</table>

aLymphocytes from BALB/C spleens sensitized against SVT2 monolayers, in vitro, and were treated with anti-mouse brain serum (1:8 dilution and guinea pig complement (1:4 dilution) prior to use in the cytotoxicity assay.

bCytotoxicity was determined by comparing the radioactivity released from the experimental cultures to that released by the freeze thawed cultures. Control cultures consisted of unsensitized lymphocytes of cultures containing only labeled target cells. These cultures consistently had 10-15% Cr$^{51}$ release. All cytotoxicities were performed in triplicate and the results represent the means ± standard deviation of 5 experiments.
TABLE 4

HOMING OF IN VITRO SENSITIZED LYMPHOCYTES
TO SYNGENEIC TUMOR GRAFTS

<table>
<thead>
<tr>
<th>Effector Cells</th>
<th>% $^{51}$Cr Localization $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVT2 cells</td>
</tr>
<tr>
<td>Sensitized Lymphocytes $^a$</td>
<td>1.8 ±0.09</td>
</tr>
<tr>
<td>Unsensitized Lymphocytes</td>
<td>1.3 ±0.08</td>
</tr>
</tbody>
</table>

$^a$Lymphocytes from BALB/C spleens sensitized against SVT2 monolayers were labelled with 50μCi of Cr$^{51}$ and injected (10$^7$ cells) into the tail veins of syngeneic recipients. The sensitized lymphocytes and the unsensitized control lymphocytes were each injected into groups of 10 animals and the results are expressed as the mean ± standard deviation of 6 experiments.

$^b$10$^5$ SVT2 cells were injected into the right footpad and 10$^5$ 4198V cells were injected into the left footpad. The mice had their legs amputated below the popliteal node and counted in a Gamma counter. Percent localization was determined by dividing the total radioactivity injected by the amount of radioactivity in the footpads.
TABLE 5

CYTOTOXIC EFFECTS OF RECRUITED LYMPHOCYTES<sup>a</sup>

<table>
<thead>
<tr>
<th>Effector cell source</th>
<th>%&lt;sup&gt;51&lt;/sup&gt;Cr release from target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVT2</td>
</tr>
<tr>
<td>Regional lymph node receiving sensitized cells</td>
<td>38.3 ± 2.8</td>
</tr>
<tr>
<td>Regional lymph node receiving unsensitized cells</td>
<td>10.2 ± 1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cytotoxicity was determined by comparing the radioactivity released from the experimental cultures to that released by the freeze thawed culture. Control cultures consisted of unsensitized lymphocytes or cultures containing only labeled target cells. These cultures consistently had 10-15%<sup>51</sup>Cr release.

<sup>b</sup><sup>10</sup>7 <i>in vitro</i> sensitized lymphocytes which had been irradiated (1000R) were injected in the right footpad of syngeneic BALB/C mice. The regional nodes were removed from the groups which consisted of 6 animals per group. The results represent the means of 5 experiments.

<sup>c</sup>Unsensitized control lymphocytes were irradiated and injected into footpads of syngeneic recipients in the same manner as the sensitized lymphocytes.
PART I

Figure 1  A phase micrograph of a thymus reticulo-epithelial cell 2 days after culture. The cytoplasm contains numerous refractile granules typical of a secretory cell. The thymocytes are out of the plane of focus.

Figure 2  A light micrograph of thymus reticulo-epithelial cells after 10 days of culture. All the thymocytes have been removed from culture and the monolayers are confluent (toluidine blue stain).
PART II

Figure 1  Single cell suspensions from C57Bl/6 thymus, cortisone resistant thymus, spleen, and lymph nodes were sensitized in vitro against allogeneic mammary tumor monolayers (H-2) for 4 days. These cells were examined for their cytotoxic potential against allogeneic tumor cells of the same antigen as the sensitizing monolayers. The cytotoxicities were performed in triplicate and the results reported as the means ± the standard deviation of 3 experiments.
LYMPHOCYTE POPULATIONS

% CYTOTOXICITY

45.5 ± 6.3

12.0 ± 2.0

22.3 ± 2.7

45.8 ± 8.9
PART II

Figure 2  A diagrammatic representation of T-lymphocyte differentiation. T1 lymphocytes which are located primarily in the thymus migrate to the spleen where under antigenic stimulation they can become T2 lymphocytes and migrate to the lymph nodes. A small population of T2 lymphocytes are also contained in the thymus and can migrate directly to the lymph nodes.
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of self recognizing lymphocytes. J. Exp. Med., 137:


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<th>Authors</th>
<th>Reference</th>
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