Title: Comparison of Thymic Cells in the Developing and
Involuting Rat Thymus In Vitro

Abstract approved: Professor Ernst J. Dornfeld

In the rat thymus, four stages of development have been distinguished: (1) a newborn stage (Stage I), (2) an accelerated growth stage (Stage II), (3) an involuting stage (Stage III), and (4) an involuted stage (Stage IV). There were three major types of thymic lymphoid cells: small (STL), medium (MTL), and large (LTL) thymic lymphoid cells. By Ficoll-Hypaque gradient centrifugation, MTL- and LTL-rich populations were isolated for comparative analysis.

Growth kinetics studies revealed that the MTL were the most active in proliferation and the most responsive to Concanavalin A (Con A) activation. The LTL had lower Con A responsiveness, and the STL were the least active in both categories. Stage II cell populations were found to be the most proliferative and Con A reactive. Stage I and III cell populations were second in both categories of cellular response, while Stage IV cells were the least active in both proliferation and Con A activation. Stage III and IV cells also appeared to be
more tolerant of high serum concentration than Stages I and II cells. Increases in cell concentration had synergistic effects on cellular proliferation and responsiveness to Con A activation. The suggested mechanism involves cell-to-cell mediation requiring intimate cell contact and possibly the formation of gap-junctions. The newborn MTL can tolerate a limited range of low temperature, and this tolerance is lost rapidly as a rat matures. On the other hand, an older rat has a higher proportion of long-lived MTL in its thymus.

The proportion of reticuloepithelial cells (REC) decreased with age in the rat thymus, while the proportion of free macrophages remained unchanged. The number of lipoid cells increased with age, and was the result of REC and macrophage differentiation. Non-lymphoid cell and lymphoid cell rosettes in thymus explants were interpreted to be interacting macrophages and thymic lymphoid cells leading to the differentiation of the latter. Macrophages also served to remove debris and to restructure the involuting thymus.
Comparisons of Thymic Cells in the Developing and Involuting Rat Thymus \textit{In Vitro}

by

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COMPARISONS OF THYMIC CELLS IN THE DEVELOPING
AND INVOLUTING RAT THYMUS IN VITRO

INTRODUCTION

In the rat, the thymus is a roughly triangular-shaped lymphatic organ which lies in the thorax immediately beneath the top of the sternum. Its base rests on the pericardium and its apex extends into the neck. It consists of two major lobes which are divided symmetrically along the midline.

Detailed investigations of the thymus were started at the turn of the century by Beard (1900, 1902), Bell (1906, 1917) and many others. Although much progress was made, misconceptions were many especially regarding the age related weight change of the thymus. It had been popular to view the larger thymus of young children as a form of pathological hypertrophy, and much useless and dangerous irradiation of the thymus was performed (Clark, 1955; Simpson et al., 1957). This practice was not stopped until Hammar (1926, 1929), and Boyd (1927, 1932) provided data on the range of thymus weights from prematurity to old age. Unfortunately, the shattering of a long held concept and practice seemed to make the study of the thymus unpopular and distasteful for a decade. Although much was known of the gross anatomy, histology and pathology of the thymus, the usual textbooks, even during the early 1960's, had no more than a brief description of
the thymus, and its role in the body was usually described as unknown. The same kind of ignorance also prevailed concerning the major cell type in the thymus—the thymic lymphocyte.

The thymus remained somewhat of an enigmatic organ until about 16 years ago when Miller (1961), Good et al. (1962), and Jankovic et al. (1962) performed a series of experiments which revealed that the thymus plays a major role in the development of the immune system. These scientists thymectomized mice at birth and at various intervals thereafter. They found that thymectomized neonatal animals showed marked depletion of lymphocytes and serious impairment of immune response to injected cellular antigens and to skin allografts. Thymectomized older mice showed little direct effect, but after lethal irradiation they were unable to regain lymphocyte-forming and immunologic functions, even with injections of bone marrow cells. However, non-thymectomized but irradiated older mice could regain their lymphocyte population and immunological function after injections of bone marrow cells. Miller (1961) concluded that the thymus was necessary for the establishment and maturation of immunological function in the newborn mouse, and in the adult it was responsible for the maintenance and reestablishment of immunological activities.

One of the major advances in the biology of immunity in the past two decades has been the recognition that lymphocytes are central to
immune activities. It is now generally accepted that a class of precursor lymphocytes from the bone marrow migrates to the thymus where these cells develop certain specific characteristics and immune functions by virtue of some crucial influence of the thymus (Katz, 1977). These thymus derived lymphocytes, referred to as T lymphocytes, are the principal cell type involved in cell mediated immunity. The T lymphocytes migrate from the thymus out into the various peripheral lymphoid tissues to play a major role in immunological surveillance (Burnet, 1970) by participating in (a) the resistance against viruses, fungi, and intracellular bacteria, (b) delayed hypersensitivity reaction, (c) graft rejection, (d) tumor immunity, and (e) regulation of immune responses (Good and Gabrielson, 1964).

Microscopically, the thymus is surrounded by a capsule of connective tissue derived from the mesenchyme. The lobes are further divided into incomplete lobules by connective tissue extending from the capsule. The peripheral part of each lobule, termed the cortex, is heavily infiltrated by cells of the lymphatic series, while the more central part of the lobule, called the medulla, has much fewer lymphoid cells. Cells of the lymphatic series in the outer cortex are large, those in the inner cortex are small in size, while the ones in the middle are of the medium variety. Light microscope studies conducted by Sainte-Marie et al. (1958, 1964, 1970, 1971) suggest that mitosis occurs in the largest and the medium-sized cells,
and that the largest lymphoid cell probably gives rise to 128 small thymic lymphocytes. Their findings also indicate that as cells multiply and differentiate to form small thymic lymphocytes, the latter move through the reticuloepithelial network and into the medulla where, through diapedesis, they enter into the lumen of blood vessels and into the circulation. Morphological details of the earlier events which involve the entry of lymphoid cell precursors into the thymus and the production of lymphoblasts from these cells are unknown.

Good and his co-workers (1964) confirmed that involution of the thymus begins around the time of sexual maturity in mice and humans. Makinodan's work on aging suggests that certain normal immunological activities declined with thymus involution. This has prompted many (Adler et al., 1971; Konen et al., 1973; Hori et al., 1973; Kishimoto et al., 1969; Goodman and Makinodan, 1975) to investigate the cellular involvement in age-related decline of immune function, and the results show that the decline is closely related to changes in the T cell population. Since thymus involution and immunological decline are both age-related events, it has been suggested (Bach et al., 1973; Hirokawa and Makinodan, 1975) that the involuted thymus loses its ability to transform precursor cells into T cells.

Although the literature on the mouse and human thymus is voluminous, many cellular aspects of the rat thymus have yet to be investigated. A series of base-line studies on age-related sequential events
of thymuses from a well documented rat colony can add to the overall picture of the mechanism of development and senescence of the immune system. Such studies can also provide the basic immunodevelopmental information with which further investigation on the rat thymus can be conducted. An age-related comparison of newborn, involuting and involuted thymuses is important because associated with the decline of the immune system is an increase in the incidence of certain types of infection, autoimmunity, and cancer (Walford, 1969). Since thymus involution and immunological decline are possibly intimately related events, any addition of information about the former may help to bring about the arrest of the latter.

The present study was undertaken to compare, by modern in vitro methods, the self-proliferating characteristics and immunocompetence of thymic lymphoid cells from newborn to involuted rat thymuses. Soluble Concanavalin A (Con A), a plant lectin which selectively activates T-lymphocytes, was used as an indication of the presence of T-lymphocyte-like cells. The cell populations studied were (a) the mechanically dissociated thymic lymphoid cell population rich in small lymphoid cells and (b) a population enriched with medium and large thymic lymphoid cells isolated by Ficoll-Hypaque gradient centrifugation. $^3$H-Thymidine incorporation by cultured cells was used as an assay of the level of self-proliferation and Con A activation. In particular, this study has sought to relate the
characteristics of different thymic lymphoid cell types to particular stages of thymus development. Non-lymphoid thymic cells were studied concurrently so as to reveal any age-related \textit{in vitro} phenomena which might have a bearing on the age dependent characteristics of lymphoid cells.
MATERIAL AND METHODS

Animals

All animals used in this project were albina rats (Harvard Strain) obtained from the colony in the OSU Zoology Department vivarium. They were mostly inbred animals whose ages were carefully documented by the author. Donor rats were killed by cervical dislocation or ether anesthesia. They were weighed, shaved and whole body disinfected by immersion in 5% Clorox solution before dissection.

Preparation of Thymus and Preliminary Cell Suspension

The upper thoracic area of the animal was sterilized with 90% alcohol solution and V-incision was made at the top of the sternum. The thymus, aseptically removed in its entirety from the anterior mediastinum, was placed in a preweighed Stender-Dish containing sterile culture medium 199 (Difco). Thymus weight was determined by reweighing.

The thymus was washed twice with culture medium and cut into small pieces which were forced and washed through sterile gauze. The cell suspension collected was washed four times and designated as pre-Ficoll cell population (PFCP).
Preparation of Ficoll Isolated Cell Suspension

The Ficoll gradient cell separation method which utilizes the aggregating property of Ficoll and the high density-low viscosity property of Hypaque for cell separation was adapted from Boyum (1968); Fotimo et al. (1971), and Ritts (1975). PFCP was resuspended in culture medium Hepes 199. Ficoll-Hypaque solution, hereafter called F-H, was prepared by mixing 24 parts of 9% Ficoll (Pharmacia, Uppsala, Sweden) with 10 parts of 34% sodium diatrizoate (Hypaque, Winthrop Laboratories). Three ml of PFCP in cold Hepes 199 were carefully layered onto 3 ml of cold F-H without mixing the two. This preparation was centrifuged at 400 x g for 45 minutes at room temperature. The cellular band at the F-H culture medium interface was carefully aspirated with a Pasteur pipette. This aspirate was washed four times by centrifuging at 50 x g for 10 minutes and resuspending the cell pellet in fresh Hepes 199. All glassware which came into contact with the samples was siliconized before use.

Culture of Cell Suspension

The concentrations of cell suspensions were determined by hemocytometer counts, and the viabilities of the cells were tested by the Trypan blue exclusion method. The final concentration was adjusted to 0.5 x 10^6 cells per ml with Hepes 199 medium containing 25% heat deactivated fetal calf serum (Gibco). 1 x 10^5 lymphoid cells
(0.2 ml of cell suspension) were placed in each well of a Linbro tissue culture plate (FB-48-TC, Linbro Scientific Co.). Con A (Sigma) was added to each well, at a concentration of 2.5 ug per well. No Con A was added to the controls or to self-proliferation experiments. Three wells were used for each assay. The culture plate was incubated for 42 hours at 37°C in a humidity chamber. Eighteen hours before harvesting, one uCi of ³H-thymidine with a specific activity of 2.0 Ci per mmole (New England Nuclear) was added to each well. Some of the specifications given were modified as described subsequently to conform to specific experiments.

**Harvesting of Cell Suspension Cultures**

Eighteen hours after pulsing, cells were harvested on a cell harvester (Strong et al., 1973) modified and constructed by the author. The cell harvester had a capacity of harvesting two samples per minute. Samples were aspirated onto glass microfiber discs (Whatman GF/C) contained in 13 mm Swinnex filter holders (Millipore). Suction was provided by a vacuum pump which was calibrated at 36 mm Hg. Each well was rinsed four times with 0.8% phosphate buffered saline solution (pH 7.5). Each disc was rinsed with one ml of 10% trichloroacetic acid solution, and followed with five ml of 0.8% saline solution. The discs were pinned onto a premarked grid to air dry. A separate plate was set up to allow final viability assessment and
morphologic evaluation of non-activated and Con A activated cultures.

**Liquid Scintillation Counting**

Air dried filter discs were placed in liquid scintillation vials with five ml of 0.8% BBOT (Sigma) in toluene. Each sample was counted in a Packard Liquid Scintillation Counter Model 3310 for 20 minutes at 60% gain and 50-900 discrimination. Counting efficiency was calculated to be 22%. Results were expressed as counts per minute (cpm) per culture. Counts from background and controls were subtracted from all results.

**Experiments on the Effects of Con A Concentration**

Con A was added at concentrations of 1 ug, 2.5 ug, 5 ug, 10 ug, 15 ug and 20 ug per well. Each Con A concentration was determined in triplicate with appropriate controls.

**Experiments on the Effects of Serum Concentration**

Cells at higher concentrations than previously described were pipetted into each well. Heat deactivated fetal calf serum (Gibco) was then added to each well to final serum concentrations of 5%, 10%, 25%, 50% and 75%, and a final cell concentration of 0.5 x 10^6 cells per well. Each serum concentration was performed in triplicate with appropriate controls.
Experiments on Culture Kinetics

Cultures were activated with 2.5 ug of Con A and pulsed with $^3$H-thymidine at the beginning of incubation. No Con A was added to proliferation studies. Samples were harvested at intervals of 1 hour, 3 hours, 6 hours, 12 hours and 24 hours. Each time interval was determined in triplicate with appropriate controls.

Experiments on the Effects of Cell Concentration

Triplicate samples were cultured at cell concentrations of $0.25 \times 10^6$, $0.5 \times 10^6$, $1.0 \times 10^6$, $2.0 \times 10^6$, $4.0 \times 10^6$, $6.0 \times 10^6$ and $8.0 \times 10^6$ cells per ml. Appropriate controls were also performed. In a separate experiment, supernatant from 24-hour-old $4.0 \times 10^6$ cells per ml FCP cultures were added to $0.5 \times 10^6$ cells per ml FCP cultures at concentrations of 10% supernatant and 50% supernatant per culture.

Experiments on the Effects of Temperature

Cultures of FCP in dram vials were separately incubated at $34^\circ C$, $37^\circ C$ and $40^\circ C$. Trypan blue exclusion tests for viability were also performed at the end of the incubation period.

Experiments on the Effects of Long Term Cultures

One set of FCP cultures was pulsed at 48 hours and harvested
at 64 hours after initial incubation. A second set of FCP cultures was pulsed at 120 hours and harvested at 138 hours after initial incubation. Residual Con A responsiveness was expressed as the ratio of 64 hour culture/138 hour culture.

Microscopic Slide Preparation and Examination

Ten drops (depending on concentration) of cell suspension were placed in each well of the cytocentrifuge (Centospin, Shandon). Samples were spun at 70 x g for five minutes. Slides were removed from the centrifuge, air dried and stained with Giemsa stain (Galigher and Kozloff, 1971). Microscopic examination was made with an oil immersion objective. Cell size was determined by means of a calibrated ocular micrometer. Differential cell counts were made by utilizing a differential blood cell counter.

Preparation of Fluorescent Labelled Con A

Fluorescent labelled Con A was prepared according to the technique described by Rinderknecht (1960, 1962). Con A was conjugated with fluorescein thioisocyanate (FITC) by shaking a mixture of 25 mg of Con A (Sigma) with 25 g of FITC on Celite (Calbiochem) in bicarbonate buffer (pH 8.8) for three minutes. The mixture was centrifuged and the supernatant was chromatographed on Biogel P-2 (1.5 x 50 cm column) to remove free FITC. The FITC-Con A conjugate was dialysed
several times against Heps 199 culture medium before use. Concentration of FITC-Con A was determined by assaying for protein concentration (Lowry et al., 1951).

**Labelling of FCP Cultures With FITC Con A and Fluorescence Microscopy**

One ml of cell suspension (0.5 x 10⁶ cells per ml) was incubated with 25 ug of FITC-Con A at 37°C for 30 minutes. Cells were then washed twice in cold phosphate buffered saline (PBS) at pH 7.3. Ten drops of suspension were placed in each well of the cytocentrifuge and spun at 70 x g for five minutes. Slides were air dried or fixed with 2% formaldehyde or glutaraldehyde for 15 minutes. One drop of 1:1 PBS/glycerin was placed on the monolayer of cells on the slide. A cover slip was affixed and sealed with wax. Slides were stored in the dark until ready to be examined in a darkened room with a Zeiss fluorescence microscope containing a reflected UV light source. Photography was performed with an Olympus (PM-6) photomicrographic camera and Kodak Tri-X film exposed for 15 minutes for separated cells and one minute for cell aggregates, using reflected UV illumination. Phase contrast micrographs were also taken for comparison.

**Explant Cultures**

After removal of surface connective tissue from the thymus,
selected regions were cut into pieces approximately 1 mm$^3$. Four pieces were carefully placed into a 25 cm$^2$ tissue culture flask (Corning Glass Works) containing three ml of Hepes 199L culture medium. All explant cultures were incubated at 37°C and culture medium was changed partially (70%) every five days. For some cultures, lymphoid cells were removed by repeated washing and replacement of fresh medium. Cultures were examined with a Wild inverted-optics phase-contrast microscope at designated time intervals. Differential cell counting was facilitated by employing an ocular grid. Phase contrast photomicrographs were taken on Kodak photomicrography monochrome film SO-410. Cells were also qualitatively determined for lipid content by staining with a saturated Sudan III solution (Galigher and Kozloff, 1971) for one hour after 24 hours of fixation with 10% formalin. Stained specimens were mounted in glycerol.

**Preparation of Culture Medium**

Hepes 199 was used in all lymphoid cell suspension cultures. It consisted of culture medium 199 in Earle's balanced salt solution without bicarbonate (Difco), and buffered with 40 mM (9.5 g/liter) (Ritts, 1975) of "Hepes" (Sigma, N-2 Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) (Chagnon and Corbeil, 1973; Shipman, 1973).

Hepes 199L was used in all non-lymphoid explant cultures. Its contents were similar to those given for Hepes 199 except for a
lower Hepes concentration of 10 mM (2.38 g/liter). Both Hepes 199 and Hepes 199L were adjusted to pH 7.2 with a 2N NaOH solution.

**Time-Lapse Photomicrography**

Time-lapse photomicrographs were taken with a Zeiss inverted-optics phase-contrast microscope and a Cine-Kodak Special II camera attached to a time-lapse control box. The culture flask being photographed was incubated with an Aircurtain Incubator (Sage Instruments) set at 37°C. The time lapse interval was set at 44 seconds per frame of film. Film used was 16 mm Kodak Tri X Reversal film 7278. The shutter of the camera was set at the 1/4 open position. The microscope objective used was Zeiss Phase I. The number of frames taken of macrophage behavior was 1909, and the number of frames taken of Stage III monolayer was 116. The processed film was studied by employing a motion analysis projector. Still photographs were made of the time lapse movie film with an Illumintron (Bogen) at intervals of one every 30 frames.

**Statistical Methods**

Counts per minute (cpm) for triplicate cultures were analyzed with respect to the mean and standard deviation(s) of the mean. Data of differential cell counts were analyzed with similar methods. Whenever two or more means had to be compared for significant difference, analysis of variance was performed. The test statistic
used was:

\[ F = \frac{s^2_{\text{from mean}}}{s^2_{\text{from individual}}} \]

The null hypothesis was rejected (indicating significant difference in the means under comparison) if the calculated \( F \) value was larger than the tabular \( F \) (\( a = 0.05 \)) at the appropriate degrees of freedom (Petersen, 1973).

**Specifications of Animals Used**

Only healthy male rats were used in this study. Animals with any external or internal ulceration or growth were rejected. Three or more rats were used for each data point determination. The ages of the animals for the different stages studied were:

<table>
<thead>
<tr>
<th>Stage I (Newborn thymus)</th>
<th>Ages of animals used (in days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage II (accelerated growth)</td>
<td>14 - 18</td>
</tr>
<tr>
<td>Stage III (involuting thymus)</td>
<td>85 - 95</td>
</tr>
<tr>
<td>Stage IV (involuted thymus)</td>
<td>260 - 270</td>
</tr>
</tbody>
</table>
List of Abbreviations:

TLC ................. thymic lymphoid cells
TNLC ............... thymic nonlymphoid cells
PFCP ................ pre-Ficoll cell populations
FCP .................. Ficoll cell populations
LTL .................. large thymic lymphoid cells
MTL .................. medium thymic lymphoid cells
STL .................. small thymic lymphoid cells
Stage I .............. newborn thymus
Stage II ............. accelerated growth
Stage III ............. involuting thymus
Stage IV ............. involuted thymus
RESULTS

Gross Examination of Thymus Growth

The growth rate of newborn to one week old rat thymuses was relatively low. The general body growth rate was faster during this period than thymus growth rate. As a result, thymus weight per gram of body weight during this period actually declined (Fig. 1). Starting from about the tenth day to the twenty-second day after birth, the thymus underwent accelerated growth to an extent that its weight increased at a much faster rate than body weight. From the twenty-fifth to the fifty-fifth day, though the thymus was still growing, the rate of growth was much reduced. The thymus started involuting at about the sixtieth day and the process of involution continued until the sixth month, after which the thymus weight stabilized. Figure 2 illustrates the appearances and dimensions of thymuses when full grown and during involution. The present study focuses on four stages of development as illustrated in Fig. 1: Stage I--newborn thymus, Stage II--fast growing thymus, Stage III--involuting thymus, and Stage IV--involuted thymus. Subsequent discussions will refer to these stages by number.
FIGURE 1.  Thymus Weight Versus Age (----), and Thymus Weight per Gram of Body Weight Versus Age (-----).

Stage I------Newborn thymus
Stage II-----Thymus undergoing accelerated growth
Stage III ----Involuting thymus
Stage IV ----Involuted thymus
FIGURE 2a. A Full Grown Thymus from a 60 Day Old Rat.

FIGURE 2b. An Involuting Thymus from a 120 Day Old Rat.
Microscopic Analysis of Pre-Ficoll Cell Populations in Suspension

Lymphoid cells dissociated from the thymuses by mechanical dispersion fell into three size classes (Fig. 3a, b): large thymic lymphocytes (LTL), medium thymic lymphocytes (MTL) and small thymic lymphocytes (STL). LTL were about 12-19μ in diameter and their nuclei were the least densely stained of the three. MTL were about 8-11μ in diameter with medium density nuclei. STL were about 5-7μ in diameter with the most densely stained nuclei. Differential cell counts (Table Ia) revealed that newborn thymuses had 16% LTL which was significantly higher (P< 0.05) than those of the other three stages. Fast growing and involuting thymuses both had 30% MTL which were significantly higher (P< 0.05) than those of the newborn and involuted thymuses. There were no significant differences in the percentages of STL among the four stages. However, STL were the most numerous (65-76%) at all four stages of thymus development.

Microscopic Analysis of Cell Populations Isolated From Ficoll Gradients

All three cell types were present in the Ficoll isolated cell population (FCP) (Fig. 3b). Differential counts (Table Ib) revealed that the percentages of STL had dropped drastically in favor of MTL and LTL (Table 1a, b). MTL constituted the most numerous cell type
FIGURE 3a. Medium (M) and Small (S) Thymic Lymphoid Cells from a Pre-Ficoll Cell Population (PFCP). (X8500)

FIGURE 3b. Large (L), Medium and Small Thymic Lymphoid Cells from a Ficoll Cell Population (FCP). (X8500)
Figure 3a

Figure 3b
Table Ia. Differential Cell Counts of Pre-Ficoll Cell Populations (PFCP), Expressed as Means of at Least Six Animals ± SEM.

<table>
<thead>
<tr>
<th>Stage of thymus development</th>
<th>LTL</th>
<th>%</th>
<th>MTL</th>
<th>%</th>
<th>STL</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>32±4.5</td>
<td>16</td>
<td>28±3.1</td>
<td>14</td>
<td>140±10.3</td>
<td>70</td>
</tr>
<tr>
<td>Stage II</td>
<td>10±3.5</td>
<td>5</td>
<td>60±5.9</td>
<td>30</td>
<td>130±9.5</td>
<td>65</td>
</tr>
<tr>
<td>Stage III</td>
<td>8±3.2</td>
<td>4</td>
<td>60±5.1</td>
<td>30</td>
<td>132±8.6</td>
<td>66</td>
</tr>
<tr>
<td>Stage IV</td>
<td>12±3.8</td>
<td>6</td>
<td>32±4.4</td>
<td>18</td>
<td>152±11.8</td>
<td>76</td>
</tr>
</tbody>
</table>

Table Ib. Differential Cell Counts of Ficoll Cell Population (FCP) Expressed as Means of at Least Six Animals ± SEM.

<table>
<thead>
<tr>
<th>Stage of thymus development</th>
<th>LTL</th>
<th>%</th>
<th>MTL</th>
<th>%</th>
<th>STL</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>94±5.7</td>
<td>47</td>
<td>94±6.1</td>
<td>47</td>
<td>12±2.1</td>
<td>6</td>
</tr>
<tr>
<td>Stage II</td>
<td>32±4.8</td>
<td>16</td>
<td>158±11.8</td>
<td>74</td>
<td>20±3.2</td>
<td>10</td>
</tr>
<tr>
<td>Stage III</td>
<td>36±5.4</td>
<td>18</td>
<td>128±9.2</td>
<td>64</td>
<td>36±4.2</td>
<td>18</td>
</tr>
<tr>
<td>Stage IV</td>
<td>69±7.1</td>
<td>35</td>
<td>85±6.3</td>
<td>43</td>
<td>46±3.9</td>
<td>23</td>
</tr>
</tbody>
</table>
FIGURE 4. Con A Activated Thymic Lymphoid Cells from Ficoll Cell Population. These Cells are Undergoing Blastogenesis During Which There is a Marked Cell Enlargement and the Nucleus is Much Less Densely Stained. (X8500)
in the FCP of fast growing, involuting and involuted thymuses. LTL and MTL were equally numerous in the FCP of newborn thymuses. However, there were significant differences in distribution of the three cell types at the four stages of thymus development (Table 1). The next aspects of this study concentrates on the comparison of pre-Ficoll cell populations (PFCP) and Ficoll isolated cell populations (FCP) at the four stages of thymus development.

Concanavalin A (Con A) is a plant lectin which in its soluble form is a mitogen which specifically activates T-lymphocytes and some sub-populations of thymic lymphoid cells. In order to identify the cell populations responsive to Con A activation and to determine whether there were age related dosage effects, the following experiments on the effects of Con A concentrations on PFCP and FCP were performed.

**Effects of Con A Concentrations on PFCP**

The effects of Con A on PFCP in terms of $^3$H-thymidine uptake are shown in Figure 5. $^3$H-thymidine uptake by the PFCP peaked at 2.5ug of Con A per culture, except those from Stage I which peaked at 5ug of Con A per culture. PFCP from Stage II had the highest response assayed at $0.48 \times 10^3$ cpm per culture, and were followed in descending order by Stages III, I and IV.
FIGURE 5. Effects of Con A Concentration on Age-Related PFCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  ●
Stage II  ○
Stage III ▲
Stage IV ▲
Effects of Con A Concentrations on FCP

The effects of Con A on FCP in terms of $^3$H-thymidine uptake are shown in Figure 6. $^3$H-thymidine uptake by FCP from all four stages peaked at 2.5ug of Con A per ml. But Stage II cells had the highest amount of incorporation at $1.7 \times 10^3$ cpm per culture which was about three and a half times the uptake of its counterpart in the PFCP. $^3$H-thymidine incorporation at the other three stages was also higher than their counterparts in the PFCP at about twice the level. However, $^3$H-thymidine uptake by the Stage II FCP was significantly higher than those of the other three stages ($P < 0.05$).

Cell growth and activation in vitro often exhibit various degrees of serum dependency. The following four experiments on the effects of serum concentration on proliferation and Con A activation were designed to investigate whether the age of the thymus influences serum requirement, and whether sub-populations of thymic lymphoid cells at different stages of development show different serum concentration preferences.

Effects of Serum Concentration on PFCP

Results for this experiment are shown in Figure 7. Optimum growth occurred at a serum concentration of 25% or higher. Although some decline was noticed in Stages II and III at higher serum concentrations, the differences were not significant.
FIGURE 6. Effects of Con A Concentration on Age-Related FCP, Expressed as $^{3}$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  •
Stage II  o
Stage III △
Stage IV ▲
FIGURE 7. Effects of Serum Concentration on Age-Related PFCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals $\pm$ SEM.

Stage I  o
Stage II  o
Stage III △
Stage IV ▲
Effects of Serum Concentrations on FCP

Results of this experiment are shown in Figure 8. Twenty-five percent serum was the preferred concentration in all four stages. Stages I and II FCP had almost twofold increases over their PFCP counterparts. However, Stages I and II FCP experienced a decline at higher serum concentrations while Stages III and IV FCP maintained their levels of $^3$H-thymidine incorporation at the higher serum concentrations. At the lower end of serum concentrations, Stages I and II FCP appeared to be more adaptable than Stages III and IV FCP.

Effects of Serum Concentrations on PFCP
Under the Influence of Con A

Results of this experiment are shown in Figure 9. Under the influence of Con A, there was about a twofold increase in $^3$H-thymidine incorporation at all four stages with Stage II experiencing the most marked increase over the PFCP cultures without Con A. The preferred serum concentration by all stages was 25%. Marked declines of $^3$H-thymidine incorporation were noticed for Stages I and II at high serum concentrations of 50% and 75%.

Effects of Serum Concentrations on FCP
Under the Influence of Con A

Results of this experiment are shown in Figure 10. Con A activated FCP from all four stages, but to different degrees. Stage
FIGURE 8. Effects of Serum Concentration on Age-Related FCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I    o
Stage II   o
Stage III  △
Stage IV   ▲
Figure 8

- CPM x 10^3 Per Culture
- % Serum In Growth Medium
FIGURE 9. Effects of Serum Concentration on Con A Activated Age-Related PFCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>●</td>
</tr>
<tr>
<td>Stage II</td>
<td>○</td>
</tr>
<tr>
<td>Stage III</td>
<td>△</td>
</tr>
<tr>
<td>Stage IV</td>
<td>▲</td>
</tr>
</tbody>
</table>
Figure 9

CPM x 10^3 Per Culture

% Serum in Growth Medium
FIGURE 10. Effects of Serum Concentration on Con A Activated Age-Related FCP, Expressed as $^{3}$H-Thymidine Incorporation per Culture in CPM $\times 10^{3}$. Symbols Represent the Mean of Three Animals $\pm$ SEM.

Stage I  •
Stage II  o
Stage III  △
Stage IV ▲
Figure 10

![Graph showing CPM x 10^3 per culture vs. % serum in growth medium.](image)

- Y-axis: CPM x 10^3 per Culture
- X-axis: % Serum in Growth Medium
II FCP exhibited a marked increase in $^3$H-thymidine incorporation—a threefold increase over the stage II FCP without Con A—at 10% serum concentration. FCP from the other three stages peaked at 25% serum concentration with Stage III, Stage I and Stage IV at descending order of $^3$H-thymidine incorporation. Declines were noted for stage I and II at higher serum concentrations of 50% and 75%.

Results presented so far were from assays of a single incubation time. $^3$H-thymidine incorporation assayed with respect to progressing incubation time yielded groups of kinetic curves which further delineated the subtle differences among lymphoid cell populations of similar morphology at different stages of thymus development. Kinetics of age related PFCP and FCP in culture with respect to cell proliferation and Con A activation were studied in the following experiments.

**Kinetics of PFCP in Culture**

The kinetics of PFCP in culture are shown in Figure 11. PFCP at Stage II had a significantly higher ($P < 0.05$) proliferation rate as reflected by its relatively high $^3$H-thymidine incorporation. Stages I and III appeared to proliferate at similar rates while Stage IV had the lowest $^3$H-thymidine uptake.

**Kinetics of FCP in Culture**

The results of this experiment are shown in Figure 12. While
FIGURE 11. Growth Kinetics of Age-Related PFCP, Expressed as $^3$H-Thymidine Incorporation for culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  ●
Stage II  ○
Stage III △
Stage IV ▲
Figure 11

CPM x 10^3 Per Culture

Hours In Culture
FIGURE 12. Growth Kinetics of Age-Related ICP, Expressed as $^{3}\text{H}$-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  •
Stage II  ○
Stage III △
Stage IV ▲
the FCP at Stage II resembled its counterpart in the previous experiment in having a significantly higher \( P < 0.05 \) proliferation rate, its maximum \(^3\text{H}\)-thymidine uptake at \( 2.89 \times 10^3 \) cpm per culture was twice as high as the Stage I PFCP. Furthermore, the Stage II FCP curve indicated that there was a burst of proliferative activity during the first three hours of culture, while Stage II PFCP showed a more gradual growth rate extending over a 12 hour period. Peak \(^3\text{H}\)-thymidine incorporation values were also higher among Stage I, III and IV FCP than their counterparts in the PFCP. However, similarity between the FCP and the PFCP were also observed—Stage II cells had the highest proliferative rate, Stage I and III cells have similar kinetic characteristics, and Stage IV cells had the lowest proliferative activity among the four stages.

**Kinetics of PFCP and FCP in Culture Activated by Con A**

There was a slight increase in \(^3\text{H}\)-thymidine incorporation by the PFCP of Stages I and IV. The magnitude of \(^3\text{H}\)-thymidine uptake by Stages II and III PFCP had significantly increased as a result of Con A activation (Fig. 13). Unlike the previous two experiments where Stages I and III kinetic curves resemble each other, while under the influence of Con A these same stages revealed two widely segregated kinetic curves for the first 6 hours in culture, indicating a difference in cellular response to Con A.
As shown in Figure 14, Stage II FCP peaked at $7.59 \times 10^3$ cpm in four hours when activated by Con A, which was a two and one-half-fold increase over its inactivated counterpart at the same period. There was a segregation of Stage III FCP and Stage I FCP kinetic curves due to the almost twofold increase of Stage III FCP $^3$H-thymidine uptake and a relatively moderate increase for Stage I cells. Even with a twofold increase in $^3$H-thymidine incorporation, Stage IV FCP had the lowest degree of activation. In general, Con A activated the FCP 2-3 times more effectively than the PFCP.

A full grown thymus contains tightly packed lymphoid cell populations whereas lymphoid cells in a very young thymus and involuted thymus have a more scattered appearance. The tightly packed cells in the full grown thymus might simply be the storage of a large number of cells, or this close contact of a large number of cells could have a functional significance. The following experiments were conducted to evaluate whether increased cell concentration influences cell proliferation and Con A activation, and to determine if these cell concentration dependent phenomena were age and population related.

**Effects of Cell Concentration on PFCP and FCP**

For non-Con A activated self proliferating cultures of PFCP and FCP, increases in $^3$H-thymidine incorporation were approximately proportional to the increase in cell concentration except for Stage I
FIGURE 13. Growth Kinetics of Con A Activated Age-Related PFCP, Expressed as $^{3}$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent The Mean of Three Animals ± SEM.

Stage I  ●
Stage II  ○
Stage III  △
Stage IV  ▲
Figure 13

![Graph showing CPM x 10^3 per culture over hours in culture.](image-url)
FIGURE 14. Growth Kinetics of Con A Activated Age-Related FCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  ●
Stage II  ○
Stage III △
Stage IV ▲
and II FCP where a 50% increase in the number of MTL resulted in almost a 200% increase in $^3$H-thymidine uptake (Table Ia, Ib, and Fig. 15, 16). The Stage III PFCP showed the highest magnitude of $^3$H-thymidine incorporation, followed by Stages II, I and IV. The order was quite different among FCP cultures where Stage II cells incorporated the highest amount of $^3$H-thymidine, followed by Stages I, III and IV.

**Effects of Cell Concentration on PFCP Cultures Activated by Con A**

As seen in Figure 17, the increases in $^3$H-thymidine uptake by Stage III and to a lesser extent, Stage II, exhibited an enhancement effect on Con A activation beyond the proportionate increase due to increase in cell number.

**Effects of Cell Concentration on FCP Cultures Activated by Con A**

As seen in Figure 18, peak $^3$H-thymidine incorporations by FCP cultures were over 20 times those by PFCP cultures. All four stages demonstrated marked increases in uptake, for up to $2 \times 10^6$ cells per ml not accountable by proportional increases in cell number alone. For Stages II and III, the rate of increase declined somewhat between $2-4 \times 10^6$ cells per ml and peaked at $15.1 \times 10^4$ cpm and $13.9 \times 10^4$ cpm culture, respectively. Stage I peaked at $11.8 \times 10^4$
FIGURE 15  Effects of Cell Concentration on Age-Related PFCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  •
Stage II  ○
Stage III △
Stage IV ▲
FIGURE 16. Effect of Cell Concentration on Age-Related FCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  ●
Stage II  ○
Stage III △
Stage IV ▲
Figure 16

[Graph showing the relationship between CPM x 10^3 per culture and Cells x 10^6 per ML.]
FIGURE 17. Effects of Cell Concentration on Con A Activated Age-Related PFCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x $10^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  ●
Stage II  ○
Stage III △
Stage IV ▲
FIGURE 18. Effects of Cell Concentration on Con A Activated Age-Related FCP, Expressed as $^3$H-Thymidine Incorporation per Culture in CPM x 10$^3$. Symbols Represent the Mean of Three Animals ± SEM.

Stage I  ●
Stage II  ○
Stage III △
Stage IV ▲
cpm without experiencing a decline in the rate of uptake. Peak incorporation values for Stages II, III and I occurred at cell concentrations of $4 \times 10^6$ per ml, and all three stages showed a drastic decline in $^3$H-thymidine uptake with increases in cell concentration. Stage IV FCP had a sharp break in incorporation rate which occurred at $2 \times 10^6$ cells per ml. This new rate was maintained and the final peak uptake value was observed to be $9.1 \times 10^4$ cpm at $8 \times 10^6$ cells per ml.

In order to investigate the synergistic effect on $^3$H-thymidine incorporation as a result of cell concentration, the following experiments were performed: (1) Supernatant from cultures of high cell concentration ($4 \times 10^6$ cells per ml) were added to cultures of lower cell concentration. (2) Fluorescent labelled Con A was allowed to react with FCP cultures and cell samples were studied microscopically.

The mechanism involved in the high-cell-concentration-synergistic-effect might involve a soluble factor secreted by the cultured cells. In an effort to ascertain such an activating factor, portions of supernatant from high cell concentration cultures were added to lower cell concentration cultures. $^3$H-thymidine incorporation data of the latter were compared with those of lower cell concentration control cultures. Microscopic studies of high cell concentration cultures activated by fluorescent labelled Con A were also conducted in order to provide a visual evaluation of
Con A binding characteristics which might aid in suggesting a mechanism behind the cell-concentration-synergistic-effect.

**Effects of Supernatant From High Cell Concentration Cultures**

Results of this experiment are shown in Table II. Supernatant from high cell concentration cultures (4 x 10^6 cells per ml) caused decreases rather than increases in ^3^H-thymidine incorporation at each of the four stages of thymus development.

**Microscopic Studies of FCP Cultures Activated by Fluorescent Labelled Con A**

Over 90% of cells in FCP cultures were observed to be labelled with fluorescent Con A. The highest intensity of fluorescence was associated with LTL and MTL. Though many STL were also labelled with fluorescent Con A, the labelling intensity was much less than those associated with LTL and MTL. The highest intensity of labelling was observed between cells within cell aggregates as shown in Figure 19b.

**Temperature Effects on FCP Cultures**

Some mammals show varying thermal regulatory capabilities at different stages of development. Newborn rats are hairless and might possess a less efficient thermal regulatory mechanism. Therefore, there exists a possibility that thymic lymphoid cells of
FIGURE 19a.  Phase Contrast Photomicrograph of a Cell Aggregate from a Fluorescent Con A Activated Stage II FCP Culture.  (X1800)

FIGURE 19b.  The Same Aggregate Illuminated with Reflected UV light, Showing Strong Fluorescence between Cells and some Cells not Labelled.  (X1800)
Table II. Effects of Supernatant from Con A Activated $4 \times 10^6$ Cells per ml Cultures on Con A Activated $0.5 \times 10^6$ Cell per ml Cultures, Expressed as $^3$H-Thymidine Incorporation in Counts Per Minute (CPM) ± SEM.

<table>
<thead>
<tr>
<th>Stage of thymus</th>
<th>Without supernatant</th>
<th>With 10% supernatant</th>
<th>With 50% supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>550±121</td>
<td>500±95</td>
<td>410±90</td>
</tr>
<tr>
<td>Stage II</td>
<td>820±156</td>
<td>770±169</td>
<td>590±100</td>
</tr>
<tr>
<td>Stage III</td>
<td>780±172</td>
<td>690±124</td>
<td>510±107</td>
</tr>
<tr>
<td>Stage IV</td>
<td>470±94</td>
<td>380±84</td>
<td>260±47</td>
</tr>
</tbody>
</table>
newborn and young rats might have a wider range of temperature
tolerance than cells from older animals. This experiment was
designed to investigate such a possibility.

Results for this experiment are shown in Table III. FCP cul-
tures from all stages experienced enhanced $^3$H-thymidine uptake
when cultured at higher (40° C) than normal (37° C) incubation
temperature. At low incubation temperature (34° C)) cells from
Stages II, III and IV showed a decline in $^3$H-thymidine incorpora-
tion while Stage I cells exhibited no significant difference in
uptake when compared with cultures at normal temperature (37° C).
Trypan blue exclusion tests indicated that there were no signifi-
cant differences in cell viability among the four stages at low
temperature.

Long Term Cultures of FCP

Hardiness of lymphoid cells in culture and the ability of these
cells to retain Con A reactivity in prolonged cultures might be a
function of the age of the thymus. Long term cultures of Con A
activated FCP from all four stages of thymus development were com-
pared with short term cultures. These comparisons were expressed
as ratios of long term culture versus short term culture, and these
ratios were evaluated in order to determine whether they suggested
the existence of an age dependent phenomenon of cell hardiness and
the ability to retain Con A reactivity in prolonged cultures.
Table III. Effects of Changes in Temperature on Con A Activated FCP Cultures, Expressed as $^3$H-Thymidine Incorporation in Counts Per Minute (CPM) ± SEM.

<table>
<thead>
<tr>
<th>Stage of thymus development</th>
<th>$34^\circ$C</th>
<th>$37^\circ$C</th>
<th>$40^\circ$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>590±106</td>
<td>550±121</td>
<td>610±125</td>
</tr>
<tr>
<td>Stage II</td>
<td>420±88</td>
<td>820±156</td>
<td>1300±210</td>
</tr>
<tr>
<td>Stage III</td>
<td>350±74</td>
<td>780±172</td>
<td>940±187</td>
</tr>
<tr>
<td>Stage IV</td>
<td>230±46</td>
<td>470±94</td>
<td>520±114</td>
</tr>
</tbody>
</table>
As seen in Table IV, there were significant differences in the ratio "cpm per 138 hour culture/cpm per 64 hour culture" among the four stages. Stage III FCP cultures retained the most $^3$H-thymidine uptake (58%) capability and Stage II retained the least (46%). Trypan blue exclusion tests did not indicate any significant differences in cell viability between the four stages at the end of 138 hours in culture.

**Thymus Explant Cultures**

Thymic lymphoid cells (TLC) of all sizes were the first cell types observed migrating from thymus explants. This migration was noticeable within 12 hours after an explant was culture, and reached its peak in about two days when a layer of TLC occupying an area of about 4-10 times the size of the explant could be observed (Fig. 20a). TLC did not adhere well to the culture flask and a semi-suspension was formed.

Next to appear were numerous thymic non-lymphoid cells (TNLC) bearing rosettes of lymphoid cells (Fig. 20b). A few TNLC at this stage had been observed to recruit additional numbers of TLC and subsequently formed a large TNLC-TLC complex. Attempts to characterize TLC of such complexes were not successful due to a very low yield in the number of TLC which could be isolated from the complexes. The majority of TNLC-TLC rosettes and TNLC-TLC
Table IV. Effects of Long Term Incubation on Con A Activated FCP Cultures, Expressed as $^3$H-Thymidine Incorporation in Counts Per Minute (CPM) ± SEM.

<table>
<thead>
<tr>
<th>Stage of thymus development</th>
<th>Incubation time = 64 hrs.</th>
<th>Incubation time = 138 hrs.</th>
<th>Residual Con A Responsiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>580±124</td>
<td>280±51</td>
<td>0.49</td>
</tr>
<tr>
<td>Stage II</td>
<td>870±161</td>
<td>400±74</td>
<td>0.46</td>
</tr>
<tr>
<td>Stage III</td>
<td>810±182</td>
<td>470±81</td>
<td>0.58</td>
</tr>
<tr>
<td>Stage IV</td>
<td>520±101</td>
<td>280±55</td>
<td>0.53</td>
</tr>
</tbody>
</table>
FIGURE 20a. Lymphoid Cell Migration from a Thymus Explant.
Age of Animal ---- 21 Days.
Age of Culture --- 15 Hours.
(X900)

FIGURE 20b. Thymic Non-Lymphoid Cell--Thymic Lymphoid Cell Rosettes.
Age of Animal ---- 30 Days.
Age of Culture --- 48 Hours.
(X900)
complexes usually disintegrate within 24 hours. Degeneration of TLC was very rapid after the fifth day in culture. Most TLC, degenerated cells and debris were removed by repeated washing with fresh growth medium.

Phase contrast microscopy revealed that TNLC from Stages I and II (Fig. 21a) were mostly reticuloepithelial cells (REC), some macrophages and few, if any, vacuole bearing cells positive for Sudan III (indicating fat or lipid content). TNLC from Stages III and IV (Fig. 21b) were REC macrophages and progressively increasing numbers of cells bearing fat or lipid vacuoles.

Reticuloepithelial cells (REC) were polygonal in shape when isolated and spindle-shaped in monolayers. REC contained oval nuclei, prominent nucleoli and paranuclear granules. These cells grew to confluence in 3-4 weeks. Stages I and II REC were observed to reach confluence faster than REC in Stages III and IV cultures.

Macrophages were ubiquitous in explant cultures from all four stages of thymus development. They constituted the most active and longest lived cell type. Differential cell counts revealed no significant difference in the number of macrophages at the different stages (Table V). Some macrophages from Stages III and IV contained lipid granules (Fig. 22a), and were observed to differentiate into lipoid cells and giant lipoid cells (Fig. 22b).

The number of granular reticuloepithelial cells was significantly lower in Stage IV cultures (Table V). Many of these became
Age of Animal ---- 15 Days.
Age of Culture --- 13 Days.
(X1800)

FIGURE 21b. A Cell Monolayer Grown from Explants of an Involuting Rat Thymus, Consisting Mostly of REC and Lipoid Cells (LC).
Age of Animal ---- 125 Days.
Age of Culture --- 19 Days.
(X1800)
Table V. Differential Cell Counts of Thymic Non-Lymphoid Cells in Explant Cultures, Expressed as Means of at Least Six Animals ± SEM.

<table>
<thead>
<tr>
<th>Stage of thymus development</th>
<th>Granular reticulo-epithelial cells (REC) %</th>
<th>Free macrophages %</th>
<th>Lipoid Cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>170±6.7</td>
<td>29±6.5</td>
<td>0--1 &lt; 1</td>
</tr>
<tr>
<td>Stage II</td>
<td>165±8.0</td>
<td>32±7.0</td>
<td>3±1 1.5</td>
</tr>
<tr>
<td>Stage III</td>
<td>161±9.9</td>
<td>30±6.0</td>
<td>9±3.5 5</td>
</tr>
<tr>
<td>Stage IV</td>
<td>112±13.2</td>
<td>36±7.8</td>
<td>37±6.2 19</td>
</tr>
</tbody>
</table>
FIGURE 22a. A Lipid Bearing Macrophage (LM) and a Free Macrophage (M) from a Rat Thymus Explant. 
Age of Animal ---- 270 Days. 
Age of Culture --- 8 Days. 
(X1800)

FIGURE 22b. A Giant Lipoid Cell (GLC) Bearing Paranuclear Fat Droplets and Multiple Lipid Vacuoles. 
Age of Animal ---- 263 Days. 
Age of Culture --- 19 Days. 
(X1800)
degenerative in culture much faster (within 3 weeks) than their counterparts at earlier stages. Some have been observed to differentiate into lipoid cells.

Two main types of lipoid cells were noted: (1) lipoid cells with large lipid vacuoles occupying most of the cytoplasm and (2) lipoid cells with small paranuclear fat granules. The number of lipoid cells were significantly higher in Stage IV cultures.

Time-Lapse Photomicrography of Macrophage Behavior and Stage III Thymic Non-Lymphoid Cell Monolayer

Time-lapse photomicrography of interactions between macrophages and REC monolayer revealed that a thymic macrophage could be aggressive and sometimes even invasive (Fig. 23). A macrophage was seen to enter a monolayer of TNLC, dislodge a TNLC and carve out a cavity within a monolayer. A dislodged cell was observed to be phagocytized by the same macrophage. It has not been established whether the macrophage behavior described is unique to cultured cells or actually reflect events in vivo. Time-lapse photomicrography of a monolayer revealed a very active population of macrophages within the monolayer.
Age of Animal ---- 97 Days.
Age of Culture --- 17 Days.

a. Migration of Macrophage (M) towards Monolayer (0 min).

b. Migration of Macrophage (M) towards Monolayer (22 min).

c. Dislodgement of Monolayer Cell (C) by Macrophage (M) (88 min).

d. Formation of Cavity (Ca) in Monolayer Caused by Macrophage (M) (176 min).

e. Dislodgement of Monolayer Cell (C) by Macrophage (M) (198 min).

f. Macrophage Residing in Cavity (Ca) (308 min).

g. Phagocytosis of Dislodged Cell (C) by Macrophage (M) (374 min).

h. Exit of Macrophage from Monolayer (660 min).
FIGURE 24. A Cell Monolayer Culture from a Stage III Thymus, Showing: Reticuloepithelial Cells (REC), Lipoid Cells (LC), and Macrophages (M).
Age of Animal ---- 95 Days.
Age of Culture --- 16 Days.
Figure 24

[Image of a microscopic view with labeled areas LC, M, and REC]
DISCUSSION

In the present study it was found that for the rat, there was an initial inactivity in thymus growth relative to body weight (Stage I), followed by a period of accelerated growth relative to body weight (Stage II) and subsequent gradual decline. Involution (Stage III) started at about the sixtieth day of age, and the rat thymus was fully involuted (Stage IV) by about the sixth month. The weight/age relationship agrees well with that of the mouse and human (Boyd, 1932; Shisa, 1971; and Hirokawa et al., 1975). However, Stages I and II were noticeable only when the thymus was seen in relation to body weight but not when considered by itself (Fig. 1).

Ficoll-Hypaque gradient centrifugation resulted in enrichment of samples from all stages with MTL and LTL. This may be due to either the lower buoyant density of MTL and LTL, aggregation of STL, or a combination of the former and the latter. The level of MTL and LTL enrichment in the Ficoll-isolated-cell-populations (FCP) appears to be dependent upon the relative concentrations of MTL and LTL in the pre-Ficoll-cell populations (PFCP) (Table I). Subsequent comparisons of FCP and PFCP reflect the dominating influence of MTL and sometimes LTL on the former, and a negative influence of a high concentration of LTL on the latter.

The thymus lymphoid cell (TLC) population of mice has been shown to be heterogeneous, and subpopulations have been demonstrated by
physical separation techniques which include density gradients (Zucker and Helfman, 1976) and velocity sedimentation (Fathman et al., 1975). Their findings that STL predominate in unenriched mouse TLC populations, and the relatively lower concentrations of MTL and LTL, agree quite well with the differential cell count data of rat PFCP in this study (Table I). Zuckman and Helfman (1976) indicated that the smallest cell type is predominant in the adult animal, which is in agreement with the 76% STL content in Stage IV rat thymus. They also suggested that the mouse MTL are hydrocortisone resistant and located in the medulla, and the mouse LTL are the precursors for the other two populations. By making single cell measurements, the volumes of rat LTL, MTL and STL were estimated to be $377\mu^3$, $157\mu^3$ and $101\mu^3$, respectively. A study by Zucker et al. (1976) shows that the thymic lymphoid cell population of the mouse fell into size classes of $250\mu^3$, $146\mu^3$ and $113\mu^3$. This indicates a similarity between rat and mouse STL and MTL, but rat LTL were substantially larger than mouse LTL.

Comparison of the Effects of Con A Concentrations on Age-Related PFCP and FCP

Both PFCP and FCP reacted optimally to 2.5 ug of Con A (Figures 4 and 5). However, the PFCP did not show a significant difference among stages of thymus development. This is probably due to a lack of response of STL to Con A. The more than twofold increase in $^3$H-thymidine incorporation by FCP and the high MTL content of FCP establish
MTL as being the most Con A responsive cells in the rat thymus. Even though Stage II and Stage III FCP had similar MTL concentrations (Table I), there was a significantly higher $^3$H-thymidine incorporation by Stage II MTL. This suggests a difference between Stage II and Stage III MTL, though their size and morphology are very similar. This difference could be in the form of decreased receptiveness to Con A on the cell surface or a change in intracellular environment of MTL in the involuting rat thymus (Stage III). Stobo and his co-workers (1973) indicated in their work with neonatal and adult mouse thymus cells that a full reactivity to Con A during neonatal life was achieved during the first three weeks of life. This is compatible with the findings that Stage II MTL are the most Con A responsive cells in the rat thymus.

Comparison of the Effects of Serum Concentration on Age-Related PFCP and FCP

Even though there was no significant difference in $^3$H-thymidine incorporation among the stages as a result of increase in serum concentration, FCP had an overall higher $^3$H-thymidine uptake than PFCP, which indicates that MTL and LTL were more proliferatively active than STL (Figures 6 and 7). Under the influence of Con A, serum concentration appeared to play a significant role in cell activation (Figures 9 and 10). Stages III and IV PFCP tolerated high concentrations of serum while Stages I and II PFCP preferred a range of 10% to 25% serum. Stage II FCP had the highest $^3$H-thymidine uptake, which again established MTL as the most Con A reactive cells.
Lymphocyte inducing factors have recently been extensively studied (Goldstein et al., 1975; Komuro, 1973 and 1975). These factors are a class of polypeptide hormones which promote differentiation of lymphocytes. Thymosin has been shown to enhance the mitogen response of guinea pig thymic lymphocytes (Soppi et al., 1977). Soppi and his co-workers indicated that in the immature thymic cell subpopulation, thymosin did not induce an increased mitogen response, but caused a significant inhibition in the proliferation of guinea pig thymic lymphocytes. In the more mature subpopulation, thymosin increased significantly the phytohemagglutinin (PHA) and Con A response of guinea pig thymic lymphocytes. In the present study, fetal calf serum (FCS) was heat inactivated at 57°C. There is a possibility that the thymosin or other lymphocyte inducing factor in FCS was heat stable, and 10% to 25% FCS contained the optimum level of inducing agents for the existing culture conditions. Taking into consideration the assumption that FCS contains inducing agents acting in a way similar to that described by Soppi and his co-workers, an inhibition of Stage I and II cells (less mature) and enhancement of Stage III and IV cells (more mature) by high serum concentration (Fig. 7) is consistent with the concept. However, conclusive deductions cannot be made in this case until more definitive experiments are performed, such as culturing Con A activated cells with differentially filtered fractions of FCS.

Takeichi et al. (1976) and Elfenbein et al. (1978) reported a heat stable FCS factor which was necessary for the formation of rat TLC and
guinea pig erythrocyte rosettes, but rosette formation was blocked by Con A. The authors suggested that the serum factor was an intermediary in rosette formation. If a serum factor was involved in facilitating the binding of Con A to lymphocytes, increase in serum concentration would enhance Con A activation of thymic cells. The results of the experiments on the effects of serum concentration on Con A activation in the present study favor the concept of a serum factor being involved in Con A activation of MTL (Figures 9 and 10). In addition, the results indicate that serum dependency is an age-related phenomenon in which cells from older thymuses (Stages III and IV) require a higher serum concentration for Con A activation, while cells from younger thymuses (Stages I and II) are inhibited by high serum concentration.

The Stage II FCP had a much higher \(^{3}\text{H}\)-thymidine incorporation than the Stage III FCP, despite their similar MTL concentration, which indicates the presence of a population of more Con A reactive MTL in the Stage II thymus. The Stage II FCP \(^{3}\text{H}\)-thymidine incorporation peaked at 10% serum concentration. This strongly suggests that serum dependence is an age-related phenomenon.

**Comparison of the Proliferating Kinetics of Age-Related PFCP and FCP**

In an effort to further delineate age-related subpopulations within classes of similar cells, proliferative kinetics of PFCP and FCP were investigated. Both Stage II PFCP and FCP were shown to have a large
population of rapidly proliferating cells (Figures 11 and 12). A comparison between $^3$H-thymidine incorporations and differential cell counts (Table I), reveals that MTL represent most proliferatively active thymic cells while the STL have the lowest proliferation rate. Even though Stage I FCP has a lower number (47%) of MTL than Stage III FCP (64% MTL), their kinetic curves are very similar. This is interpreted as the result of decreased proliferation rate among Stage III MTL or some very actively proliferating LTL (47% in Stage I) in the Stage I FCP. Large thymic cells in the mouse have been postulated to be precursors of the other smaller cell types in the thymus (Metcalf, 1962; Weissman, 1973), which suggests that LTC in the newborn rat thymus may indeed be actively proliferating. However, considering the initial lack of growth in the newborn rat thymus (Fig. 1), the concept of fast dividing LTL may not apply to the rat thymus. On the other hand, if the slow growth in the newborn rat thymus was due to a lack of proliferation among reticuloepithelial cells alone, then the kinetic curve of Stage I FCP would still reflect the combined influence of MTL and LTL. The Stage III MTL also appear to have a much depressed proliferating population, because with only 10% less MTL than Stage II FCP (64% vs 74%), the Stage III kinetic curve peaked at a value which was almost 70% less than that of Stage II. Both Stage IV PFCP and FCP kinetic curves suggest that cells from involuted thymuses have a much lower proliferation rate than cells from the other stages. This eliminates the possibility that there may be a small population of fast dividing cells maintaining T-lymphocyte output in the involuted thymus.
The FCP kinetic curves showed a sharp decline after 12 hours in culture while the Stages I and II PFCP kinetic curves displaced a relatively high $^3$H-thymidine uptake level up to 24 hours in culture. This suggests that a full cell compliment, including a large population of proliferatively inactive STL, is necessary for the maintenance of proliferation. Miller (1966) in his work with mouse bone marrow tissue, found a cell type with morphological resemblances to a small lymphocyte which he suggested could migrate to thymus tissue to give rise to cells in the thymic lymphatic series. A small population of small lymphoblasts with a long generation time, within a large population of STL in the PFCP, could account for the sustained $^3$H-thymidine uptake of Stages I and II beyond 12 hours. Taking into consideration that Stages I and II represent newborn and very young rats, such migration of lymphoblasts from the bone marrow to the thymus is quite conceivable. It is emphasized, however, that critical experiments are yet to be performed in order to prove or disprove the existence of these small lymphoblasts.

Con A activation kinetics for both PFCP and FCP revealed a population of very Con A responsive MTL (Figures 13 and 14). Even though Stage III FCP had only 10% less MTL than Stage II FCP, and Stage II and III PFCP had identical percentages of MTL. A comparison of the Con A activated kinetic curves of these two stages suggested that there were more Stage II cells activated than Stage III cells. This indicates a difference in Con A responsiveness of MTL relative to the age of the thymus.
even though the MTL are identical in size and morphology. This could be the result of a young thymus (Stage II) generating a larger number of medium size T-lymphocyte-like (Con A responsive) cells for the seeding of the other lymphoid organs (lymph nodes and spleen). The involuting thymus (Stage III) which is charged only with periodic maintenance of the T-cell population renders such a high population of Con A-responsive-T-lymphocyte-like cell unnecessary. The aforementioned hypothesis would also apply to the comparison of Stage I and Stage IV Con A activated kinetic curves which reflect a difference in activation level but similarity in cellular makeup.

The Stage I Con A activated PFCP (Fig. 13) kinetic curve peaked at 12 hours instead of the 4-6 hour period for the other three stages. This suggests a population of cells which react rather slowly to Con A. If these slow reacting cells in Stage I were MTL, they would constitute a slightly different subpopulation, possibly with a lower Con A receptiveness or different intracellular environment, rendering a slower reaction. Soren (1973), working with human circulating lymphocytes did indeed come across small subpopulations which enter into S phase at widely different times after phytohemagglutinin activation. However, direct participation and indirect influence of other cell types in these kinetic curves cannot be discounted.

**Comparison of the Effects of Cell Concentration on Age-Related PFCP and ICP**

The cortex of the thymus is richly infiltrated with lymphoid cells.
These cells are packed so tightly in the cortex of a full grown thymus that a thick section at the light microscope level reveals mostly lymphoid cells which almost totally obscure from view the other non-lymphoid cell types. The question being asked in this study is whether this tight packing is only incidental to the proliferation of the lymphoid cell series or does it facilitate the proliferation and the differentiation of precursors into mature T-lymphocytes, and if so, is the phenomenon age dependent?

In an effort to answer some of these questions, cells from PFCP and FCP were cultured in increasing cell concentrations. The families of age-related PFCP curves (Fig. 15) showed only proportionate increases in $^{3}$H-thymidine uptake with increases in cell concentration, and there was no significant difference ($P < 0.05$) among stages of thymus development. Age-related FCP curves (Fig. 16) showed proportionality for Stage III and IV cells. However, Stage II and Stage I cells revealed a 200% increase in proliferation with only a 50% increase in cell number (from $4 \times 10^{6}$ to $6 \times 10^{6}$ cells per ml) and the difference between the mean $^{3}$H-thymidine incorporation levels were significant ($P < 0.05$). This disproportionate increase in $^{3}$H-thymidine uptake by Stage I and Stage II FCP can be attributed to a higher percentage of cells being brought into mitosis as a result of increases in cell concentration. This synergistic effect seems to be restricted to FCP cultures from very young animals with a high concentration of MTL and LTL (Table I). Therefore it can be hypothesized that "cell packing" appears
to enhance cell proliferation among medium and large thymic lymphoid cells in newborn and young rat thymuses. By the same token, the lack of synergistic effect in PFCP cultures could be the combined result of low MTL concentration and the spacing effect of a high concentration of STL. However, more definitive experiments have yet to be performed to verify this hypothesis.

Theories of cell-to-cell communication have been widely advanced (Sheridan, 1974; De Mello, 1977; Lash et al., 1977). It is generally accepted that gap junctions can provide a structural pathway for the transfer of small molecules from cell to cell (Gilula, 1977). It has been demonstrated by Lawrence and his co-workers (1978) that intercellular communication via gap junction of a mediator that is common to both cell types may result in cross-stimulation. The synergistic effect in cell proliferation due to an increase in cell concentration could operate by a similar mechanism. This in vitro synergistic effect might even reflect an in vivo phenomenon due to a condition common to both cases, i.e. "cell packing". However, studies of a limited number of electron micrographs prepared under some unfavorable conditions did not reveal any gap-junctions between lymphoid cells.

A synergistic effect due to increases in cell concentration was very evident among FCP cultures activated by Con A (Fig. 18). There was a 15 to 20 fold increase in $^3$H-thymidine uptake by Stage II and III FCP with only a threefold increase in cell concentration (from $0.5 \times 10^6$ to $1.5 \times 10^6$ cells per ml). Stage I FCP had a 20 fold increase in
$^3$H-thymidine uptake with only a fourfold increase in cell concentration (1.0 x $10^6$ x 4.0 x $10^6$ cells per ml). It has been shown that a thymocyte-stimulating factor (TSF) produced in supernatants of murine spleen cells stimulated with mitogens confers to thymocytes the ability to respond to Con A (Chen, 1974, 1975; Sabato, 1974). The increased-cell-concentration-synergistic-effect might work by way of the same mechanism: TSF produced by a Con A activated high cell concentration FCP culture may stimulate other previously unresponsive thymic lymphoid cells. In order to test the hypothesis, supernatant portions from Con A activated high cell concentration cultures were added to Con A activated low cell concentration cultures. No enhancement was noticed in any of the low cell concentration cultures (Table II). Apparently no TSF was formed by thymic lymphoid cells.

Supernatant from high cell concentration cultures appeared to cause decreases in $^3$H-thymidine incorporation in low cell concentration cultures. This could be the result of (a) the depletion of nutrients in the added supernatant, (b) the accumulation of cytotoxic waste, or (c) the presence of a thymic lymphocyte-inhibiting factor produced by high cell concentration cultures. A lymphocyte chalone has been described by a number of investigators (Moorhead et al., 1969; Jones et al., 1970; Garcia-Giralt et al., 1970; and La Salvia et al., 1970). Houck and Irausquin (1973) presented evidence of the existence, uniquely in lymphoid tissue, of a noncytotoxic cell specific mitotic inhibitor for lymphocyte mitosis in human lymphocytes stimulated by
phytohemagglutinin in culture. The Con A stimulated high cell concentration cultures might produce a mitotic inhibitor or an actual chalone. The apparent production of a mitotic inhibitor seems to be dependent on high cell concentration, which is compatible with the chalone concept. The lack of inhibitory effects on high cell concentration cultures could be attributed to the high number of cells diluting the per cell chalone level below threshold concentration for mitotic inhibition to take place; while in the low cell concentration cultures, supernatant from the former contained an adequate chalone concentration to reach the per cell threshold chalone level to cause mitotic inhibition or to initiate entrance into an aging pathway. Existence of a chalone is not conclusive in the present study until experiments such as non-species specific inhibitions are performed.

An alternate hypothesis for the increased-cell-concentration-synergistic-effect is cell-to-cell communication. It has been shown that exposure of co-cultures of mixed cell types to a hormone specific for one cell type cause the heterologous cells to respond through a cell contact dependent mechanism (Lawrence, 1978). The cell concentration dependent synergistic effect could operate via the same mechanism with minor modifications: (1) replacing hormone with Con A and (2) physically increasing cell contact by increasing cell concentration. A cell aggregate labelled with fluorescent Con A (Fig. 19) showed strong fluorescent labelling within the cell aggregate, especially between cells. Some cells in the aggregate were not labelled. Even
though this is only indirect evidence, a model can be proposed based on the above observations. The model has Con A serving as an activating agent and an aggregating agent, and aggregation is enhanced by increased cell concentration. Unresponsive cells (non-fluorescent labelled cells in the aggregate) are activated by cell-to-cell communication initiated by Con A activated cells. The intercellular mediator should be common to both cells and in this case cyclic AMP would fit the model quite well. However, critical experiments have yet to be performed to verify the model. Electron microscopic studies of aggregates may prove or disprove the existence of gap junctions between lymphoid cells in culture.

Edelmen (1974) suggested that for Con A receptor sites on lymphocytes, an equilibrium exists between free receptor sites and receptor sites anchored to a network of submembranous microtubules. The decline of Con A reactivity observed in cell populations of older thymuses could be the result of the degeneration of these submembranous microtubules leading to a disequilibrium between free and anchored receptor sites.

Comparison of the Effects of Temperature on Age-Related Con A Activated FCP Cultures

An increase of three degrees above the normal incubation temperature of 37°C resulted in an increase in $^3$H-thymidine uptake at all stages of thymus development (Table III). Temperature-sensitive
activities on the cell surface during Con A binding have been studied by various investigators (Nicolson, 1974; Inbar et al., 1976). Change in membrane fluidity is probably the major cause of changes in Con A binding characteristics (Nicolson, 1973). However, the temperature change required to alter membrane fluidity is beyond the temperature range being considered in this experiment. The temperature effects in the present study were probably the results of temperature dependent acceleration of $^3$H-thymidine and/or shortened generation time instead of cell surface modification. The site of action was likely to be intracellular at the enzymatic level. There was no appreciable difference ($P < 0.05$) in the level of high temperature enhancement among different stages of thymus development.

$^3$H-thymidine uptake was depressed in Stages II, III and IV FCP cultures with a decrease of three degrees below the optimum incubation temperature. Stage I $^3$H-thymidine incorporation remained relatively unchanged, and it is significantly different ($P < 0.05$) from the other stages (Table III). Stage I rats were newborn hairless animals with inadequate thermoregulatory abilities. The stability of Stage I FCP at low temperature suggests that there may be a transient mechanism for low temperature adapted Con A receptor sites or low temperature adapted intracellular environment and enzymatic system in the MTL of newborn rats.
Comparison of the Effects of Long Term Age-Related FCP Cultures

Stages III and IV cells had a significantly higher residual Con A responsiveness than cells of the other two stages, which suggests that an involuting, and an involuted thymus retains a population of relatively long-lived lymphoid cells (Table IV). This is advantageous from a functional standpoint because the much reduced thymic lymphoid cell population in the involuted thymus is compensated for by a smaller but longer lived cell population. This is not to indicate that the absolute number of long-lived cells in Stages I and II are lower. Given their higher absolute cell number, Stages I and II probably have more long-lived cells. Residual response in this case indicates the proportion of long-lived cells in each animal rather than the absolute number.

$^3$H-Thymidine Incorporation and Cell Proliferation

The magnitude of $^3$H-thymidine incorporation was used throughout the study as an index of cell proliferation. Large differences in the size of endogenous thymidine pools or thymidine transport rates among cell populations might influence the magnitude of $^3$H-thymidine incorporation. This might give rise to erroneous impressions of cell proliferation rate. Though there is as yet no evidence that such large differences exist among lymphoid cell populations in the rat thymus, caution is warranted in the interpretation of $^3$H-thymidine incorporation.
Therefore, cell proliferation rates presented in this study are not viewed as conclusive quantitative data on cell proliferation but rather as relative indices with which the characteristics of similar cell populations are compared.

**Thymic Non-Lymphoid Cells (TNLC) in Culture**

Thymic non-lymphoid cells bearing rosettes of lymphoid cells (TNLC-TLC rosettes) are the most puzzling objects in explant cultures. Efforts to characterize the lymphoid cells of the rosettes have been repeatedly frustrated due to the difficulties in isolating the TLC from a rosette. These rosettes are transient structures which dissociate into individual cells after 24 hours in culture. After observing the same phenomenon in a number of different culture systems, it is concluded that this transient association of non-lymphoid cells and lymphoid cells in thymus explants is likely to reflect an *in vivo* event rather than just an *in vitro* artifact. The functional significance of TNLC-LC rosettes cannot be ascertained until the cells from the rosette are characterized.

Beller and his co-workers (1978) reported that interaction between macrophages and T-lymphocytes resulted in enhanced secretion of the thymocyte-differentiating-factor, and that this interaction was shown to require physical contact of the two cell types. Beller's findings were based on observations of macrophages and lymphocytes artificially brought together. TNLC-LC rosettes observed in the present study
could be a visualization of the aforementioned system in its natural interacting state, operating at a large scale.

As the thymus ages, the number of lipid cells increases while the number of granular reticuloepithelial cells (REC) decreases (Table V). In an involuting thymus, there is an increased tendency for macrophages and REC to differentiate into lipid cells, but the proportion of free macrophages remained relatively constant (Table V) as the thymus ages. This indicates the presence of a mechanism for the replenishment of free macrophages in the involuting and involuted thymus. On the other hand, REC suffer a net loss as a result of degeneration and lipid cell conversion.

Since macrophages represent the most active constituents of an explant culture, a question is raised as to their function besides the already mentioned macrophage-lymphocyte interaction. Their infiltration of monolayer cultures suggests that they serve to remove cell debris generated by a large number of degenerated small lymphoid cells. The significance of the apparent invasiveness and destructiveness of free macrophages revealed by time-lapse-photomicrography is still obscure. This author suggests that as a thymus ages and its lymphoid cells are depleted, its structure starts to collapse; and in this case the apparent destructiveness of the macrophages could serve to restructure the thymus for its post-involution functions.
CONCLUSIONS AND SUMMARY

The present study represents attempts to characterize rat thymic cell subpopulations with respect to the age of the thymus. The one major concept that emerges is that medium thymic lymphoid cells (MTL) play a pivotal role in rat thymus development. Though they only represent 15-20% of the total lymphoid cells in the thymus, they are the most proliferative cells. MTL are also the most Con A responsive. The proliferative population and Con A responsiveness of MTL increases from newborn to reach their zenith at about 21 days of age (Stage II) and decline thereafter. The relatively high proportion of large thymic lymphoid cells (LTL) in the Stage I thymus probably contribute to the initial increase of MTL at the expense of large thymic lymphoid cells, resulting in a lower number of LTL at Stage III.

A MTL takes on different characteristics as it matures. It has a low serum requirement for proliferation and Con A activation. However, when it matures, serum requirement increases. A young MTL's ability to proliferate and react to Con A stimulation is enhanced by high cell concentration, but a MTL in an older rat loses this high-cell-concentration-enhancement ability. A newborn MTL can tolerate a limited low temperature, and this tolerance is lost rapidly as the rat emerges from neonatany. On the other hand, an older rat has a higher proportion of longer living MTL in its thymus.

The majority of the cells in the rat thymus are small thymic
lymphoid cells (STL). The one major characteristic for this subpopulation is that they are high in number but low in activity. They are not very proliferative and are relatively unresponsive to Con A activation. A STL probably comes from an asymmetric division of a MTL. It can also be the inactive daughter cells from a MTL division which gives rise to a T-lymphocyte and an inactive cell (Miller, 1966). Most STL are destined for early degeneration. The fact that very few STL in the thymus are found to be responsive to Con A points to two possibilities: (1) some STL will become mature T-lymphocytes but the maturation process takes place after the cells have left the thymus; (2) matured T-lymphocytes are processed out of the thymus very rapidly as they mature.

The tightly packed appearance of lymphoid cells in the thymus is not just the result of accumulating a large number of cells in a small space. This "tight packing" presumably serves to enhance cellular proliferation and differentiation by facilitating cell contact.

As the thymus involutes, many reticuloepithelial cells and macrophages differentiate into lipoid cells. As a result, the reticuloepithelial cell population declines but macrophages are replenished. The macrophage may serve a dual role in the thymus; (1) to assist in the differentiation of lymphoid cells and (2) to assist in the removal of cell debris and restructuring of the involuted thymus.


