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Title: Comparison of Thymic Cells in the Developing and

Involuting Rat Thymus In Vitro

Abstract approved:

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In the rat thymus, four stages of development have been dis-

tinquished: (1) a newborn stage (Stage I), (2) an accelerated growth

stage (Stage II), (3) an involuting stage (Stage III), and (4) an invo-

luted 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 prolifera-

tion 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 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 volumi-
nous, many cellular aspects of the rat thymus have yet to be investi-
gated. 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 immuno-developmental 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 immuno-competence 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 in vitro

phenomena which might have a bearing on the age dependent charac-

teristics of lymphoid cells.

MATERIAL AND METHODS

Animals

All animals used in this project were albino 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 pipetee. 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 sampes 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 0.5×10^6 cells per ml with Hepes 199 medium containing 25% heat deactivated fetal calf serum (Gibco). 1×10^5 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 incu-

bated for 42 hours at 37°C in a humidity chamber. Eighteen hours

before harvesting, one uCi of ^{3}H-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 subse-

quently 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% tri-

chloroacetic 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 0.5×10^6 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 0.25×10^6, 0.5×10^6, 1.0×10^6, 2.0×10^6, 4.0×10^6, 6.0×10^6 and 8.0×10^6 cells per ml. Appropriate controls were also performed. In a separate experiment, supernatant from 24-hour-old 4.0×10^6 cells per ml FCP cultures were added to 0.5×10^6 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°C, 37°C and 40°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 Hepes 199 culture medium before use. Con-

centration 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 10^6 cells per ml) was incu-

bated with 25 ug of FITC-Con A at 37^oC 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) photo-

micrographic 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 inter-

vals. 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 quali-

tatively 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 Rever-

sal 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 pro-

cessed 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:

	Ages of animals used (in days)
Stage I (Newborn thymus)	1 – 5
Stage II (accelerated growth)	14 – 18
Stage III (involuting thymus)	85 – 95
Stage IV (involuted thymus)	260 – 270

List of Abbreviations:

TLC . . . . thymic lymphoids 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
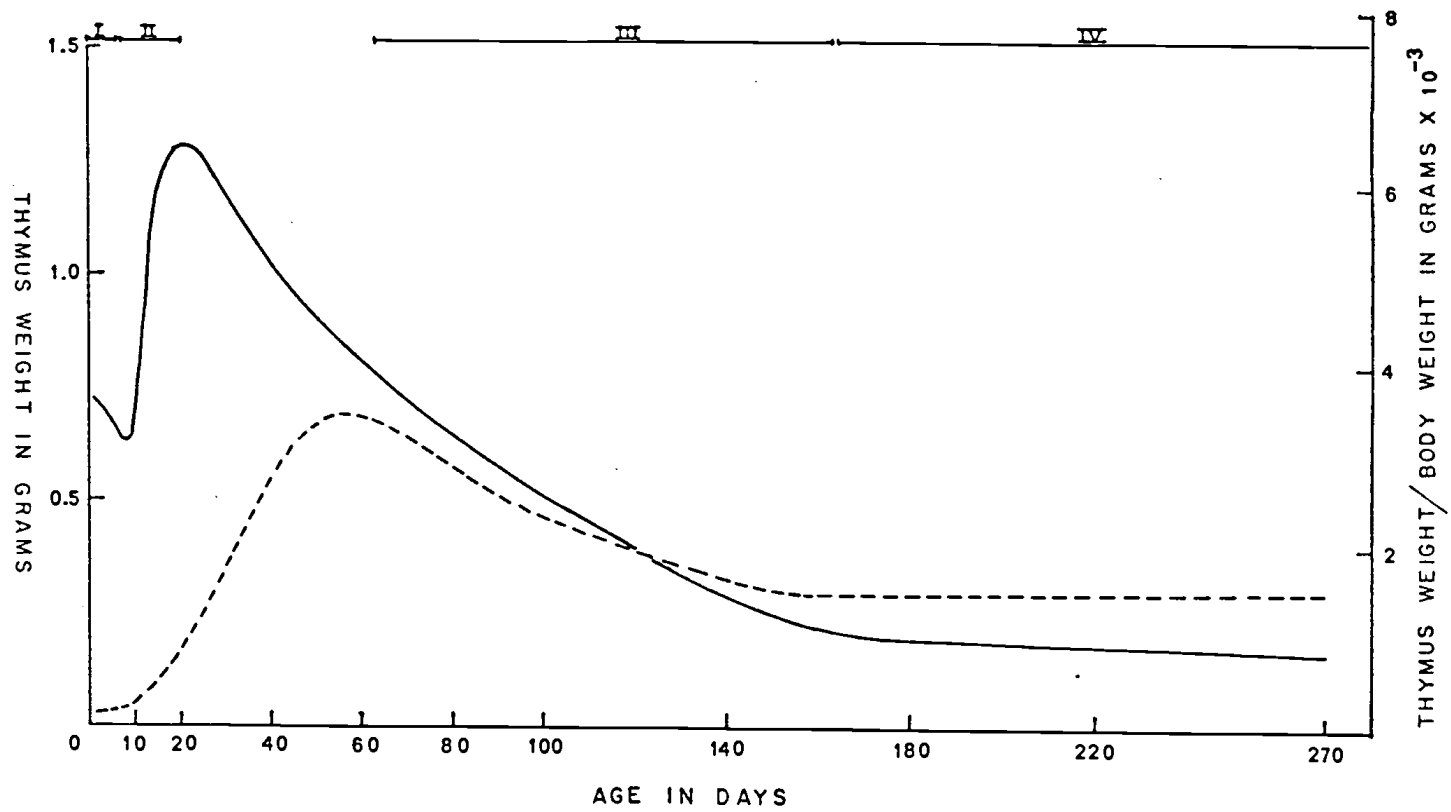
Figure 1

FIGURE 2a.   A Full Grown Thymus from a 60 Day Old Rat.

FIGURE 2b.   An Involuting Thymus from a 120 Day Old Rat.

Figure 2a
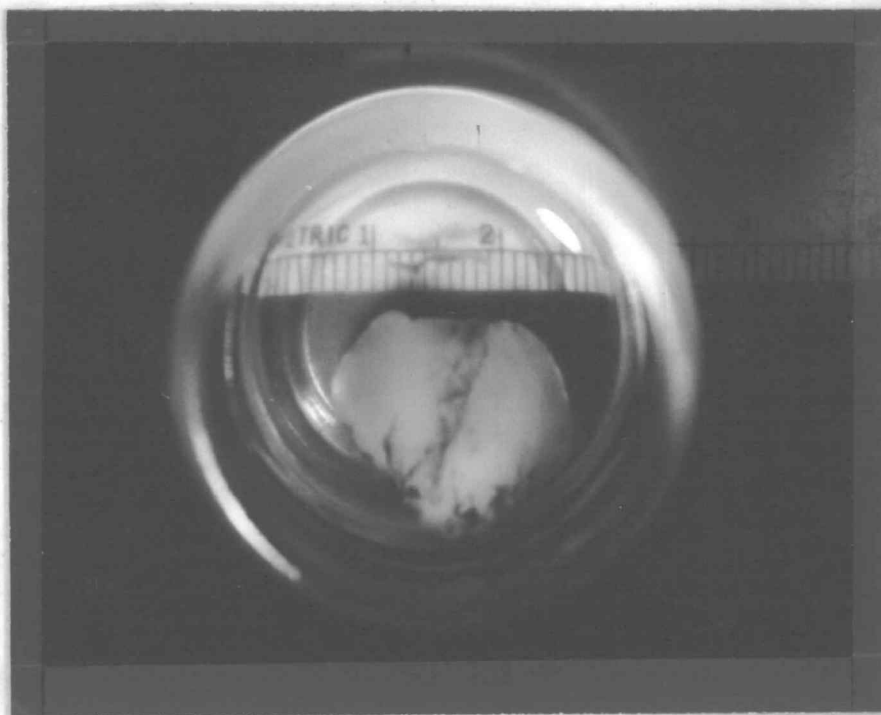
Figure 2b
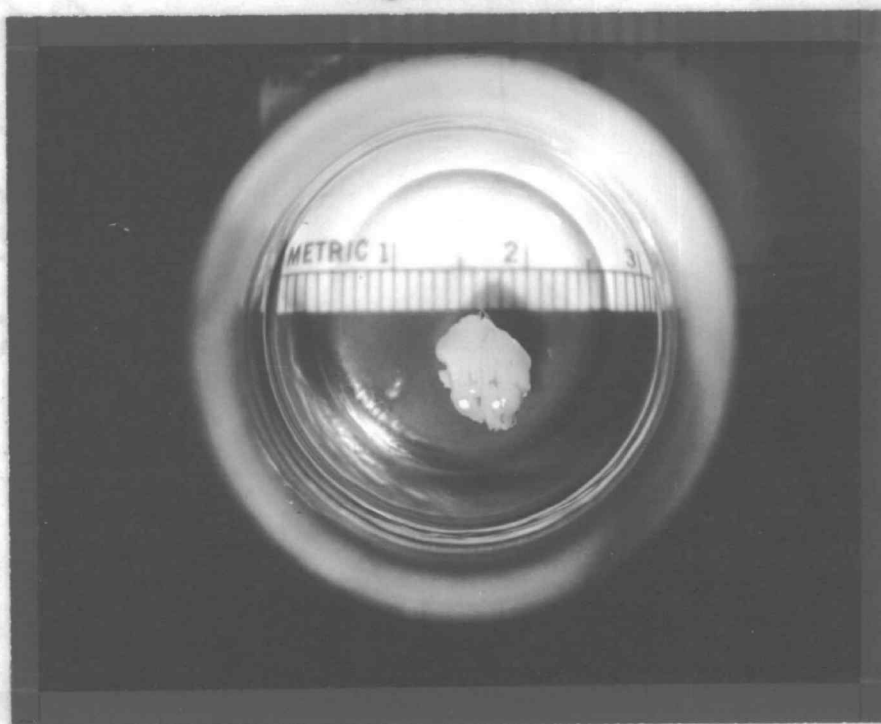
Microscopic Analysis of Pre-Ficoll
Cell Populations in Suspension

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 12-19u in diameter and their nuclei were the least densely stained of the three. MTL were about 8-11u in diameter with medium density nuclei. STL were about 5-7u 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 la,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
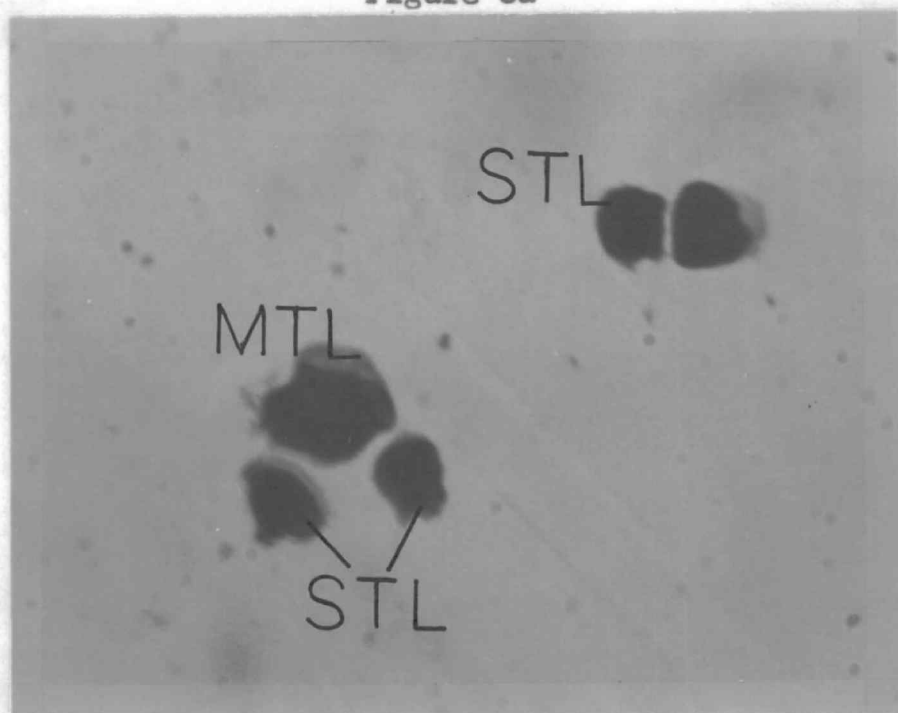
Figure 3b
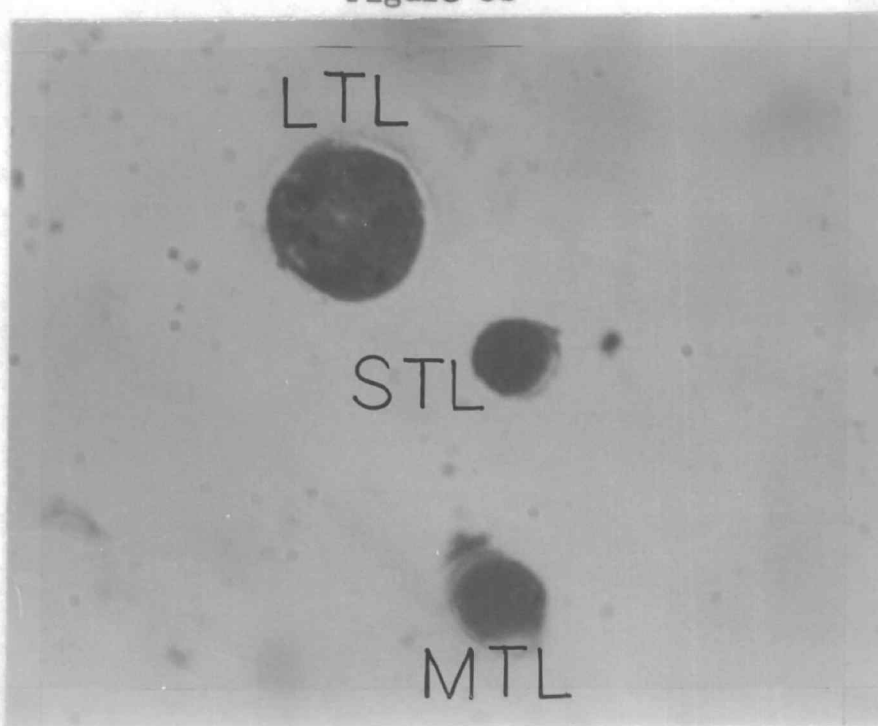
Table Ia. Differential Cell Counts of Pre-Ficoll Cell Populations (PFCP), Expressed as Means of at Least Six Animals ± SEM.

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

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

Stage of thymus development	LTL	%	MTL	%	STL	%
Stage I	94±5.7	47	94± 6.1	47	12±2.1	6
Stage II	32±4.8	16	158±11.8	74	20±3.2	10
Stage III	36±5.4	18	128± 9.2	64	36±4.2	18
Stage IV	69±7.1	35	85± 6.3	43	46±3.9	23

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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)

Figure 4
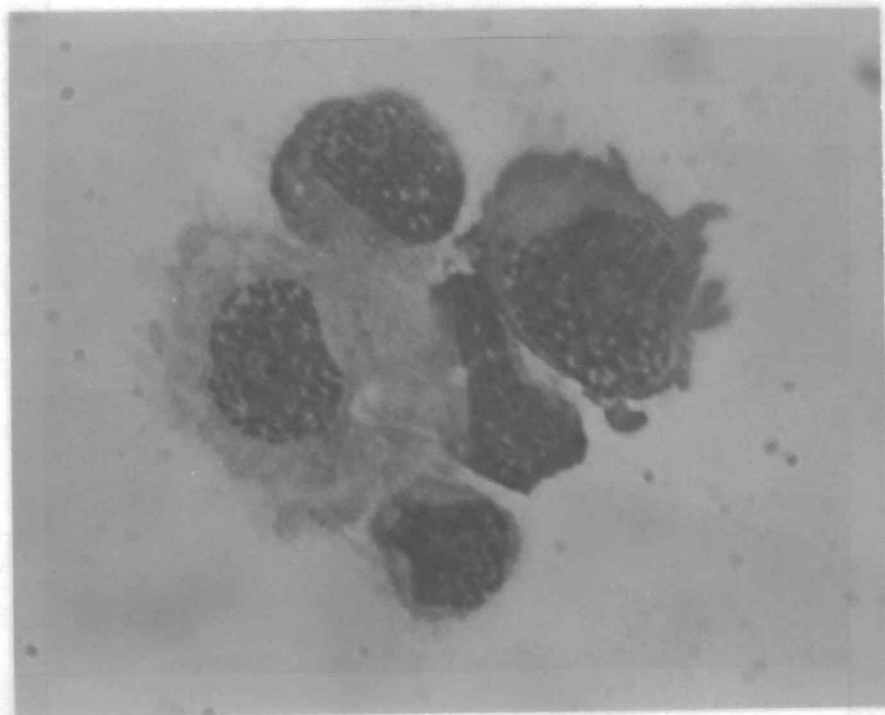
in the FCP of fast growing, involuting and involuted thymuses. LTL

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 popula-

tions (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 experi-

ments 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 re-

sponse assayed at 0.48×10^3 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 $\pm$ SEM.

Stage I   ●
Stage II   o
Stage III △
Stage IV ▲

Figure 5

CPM X 10³ Per Culture

μg Con A Per Culture

30

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 1.7×10^3 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 $\pm$ SEM.

Stage I   ●
Stage II   o
Stage III   △
Stage IV   ▲

Figure 6

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   ▲

Figure 7

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 $\pm$ SEM.

            Stage I   o
            Stage II   o
            Stage III   ⬭Δ
            Stage IV   ▲

Figure 8

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 $\pm$ SEM.

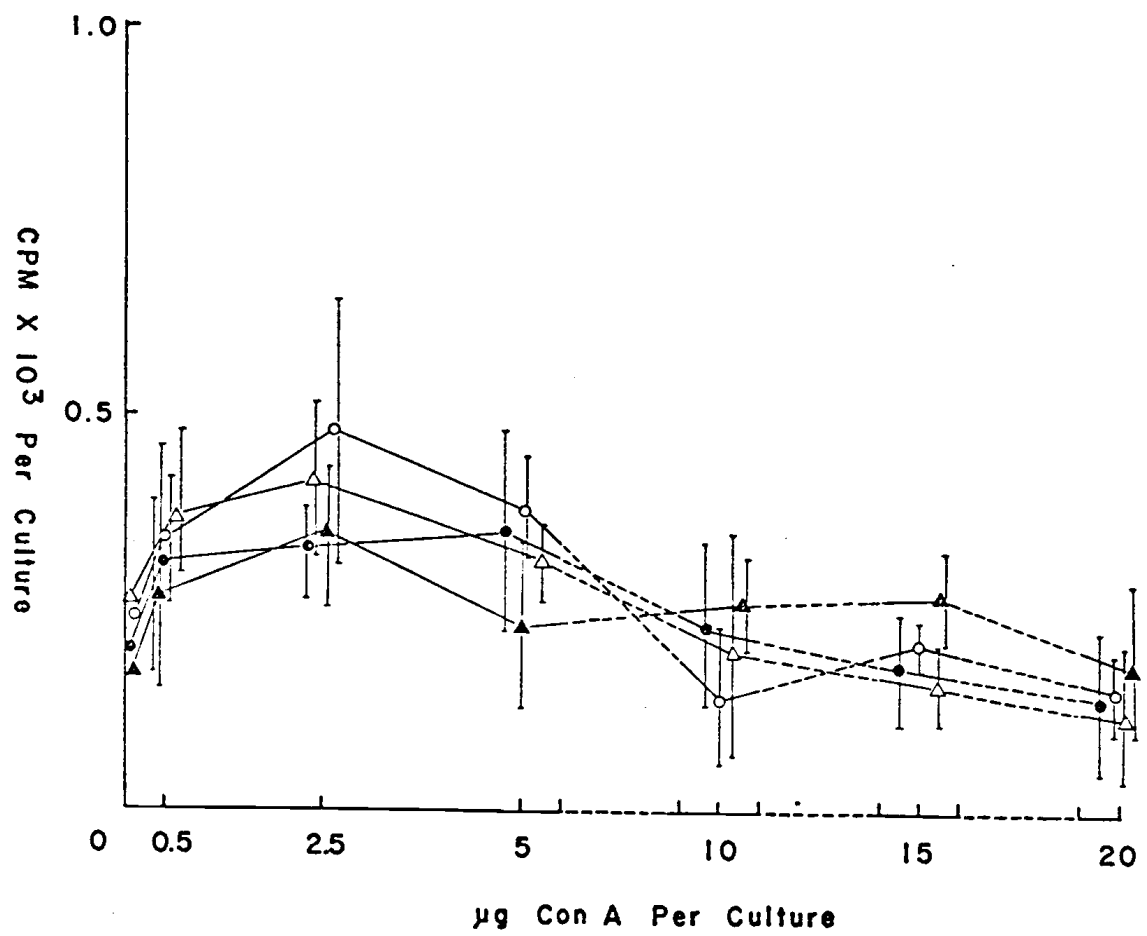Stage I   ●

Stage II   o

Stage III   △

Stage IV   ▲

Figure 9

FIGURE 10. Effects of Serum 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   o
Stage III   △
Stage IV   ▲

Figure 10

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   o
Stage III   △
Stage IV   ▲

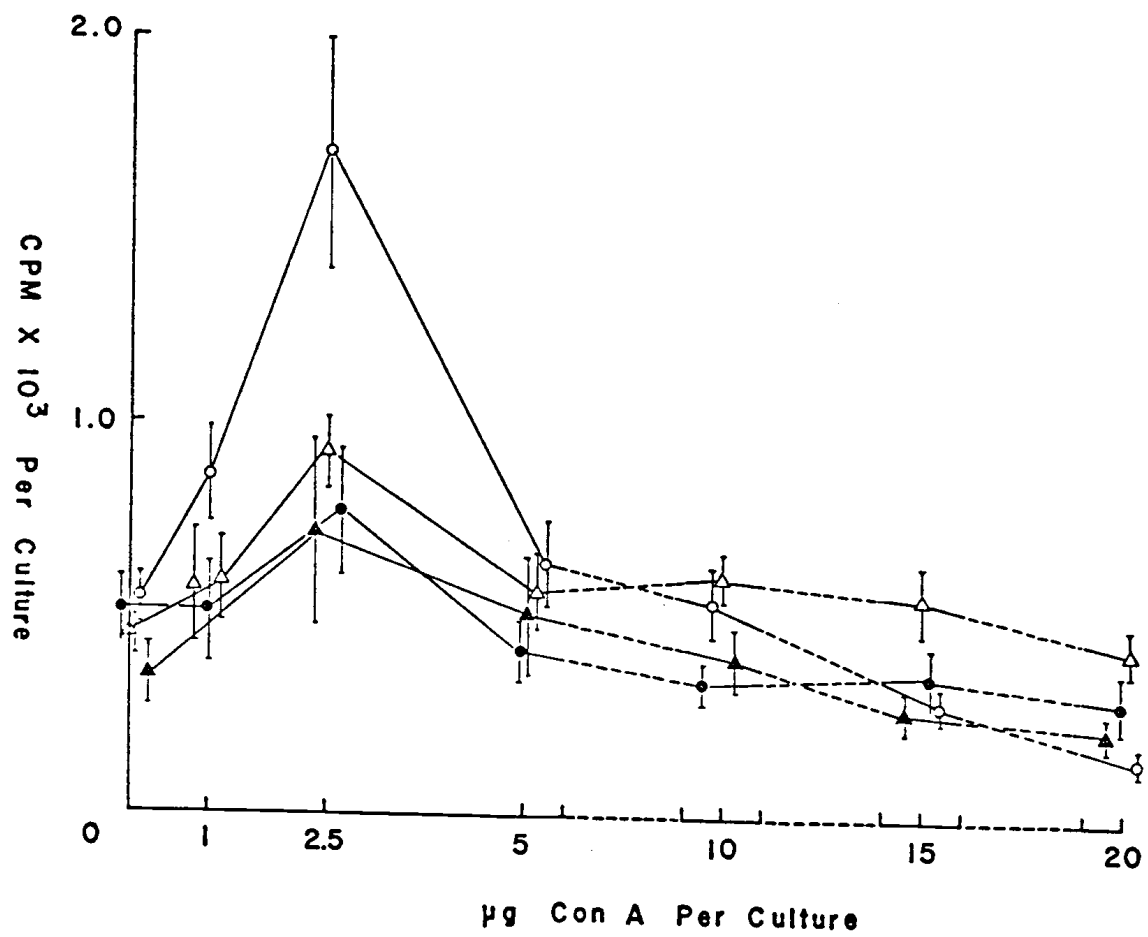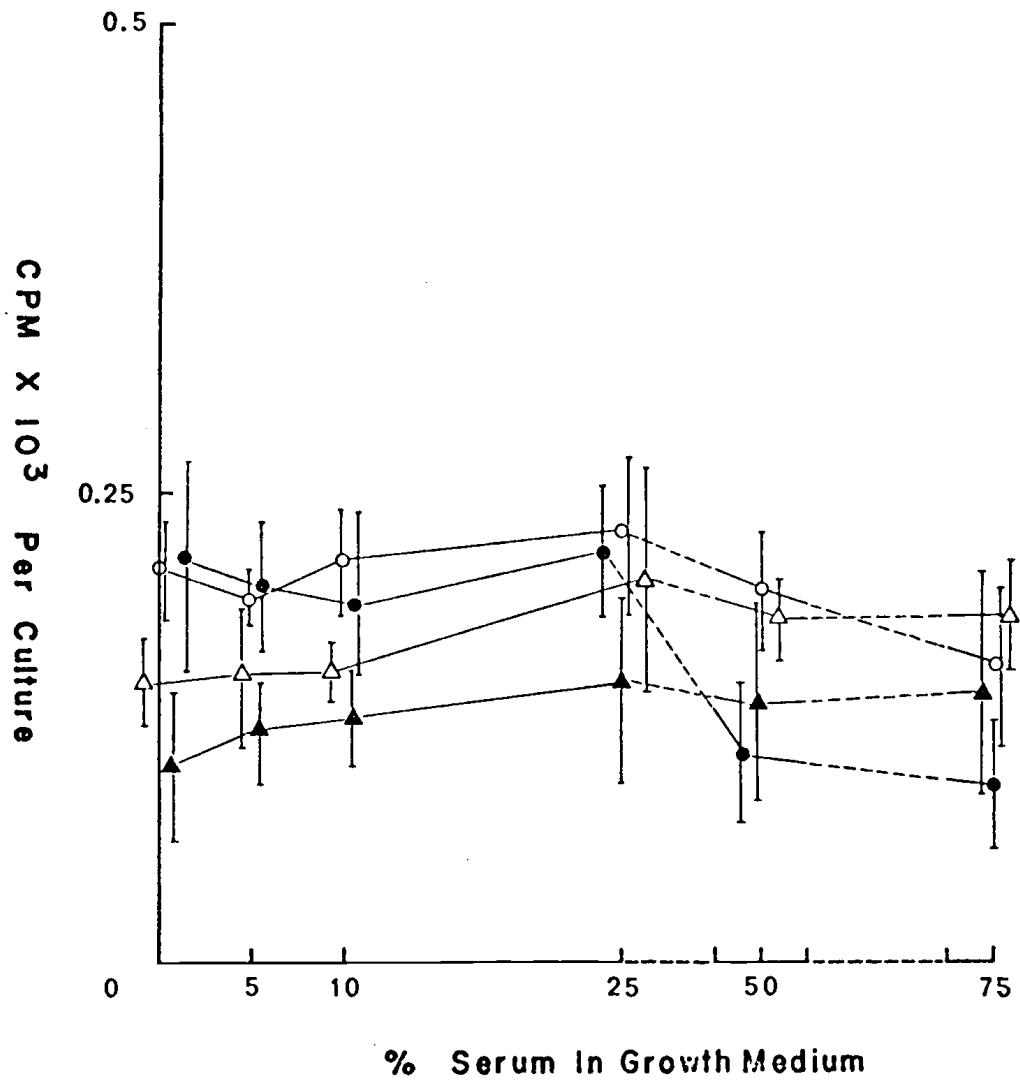Figure 11

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

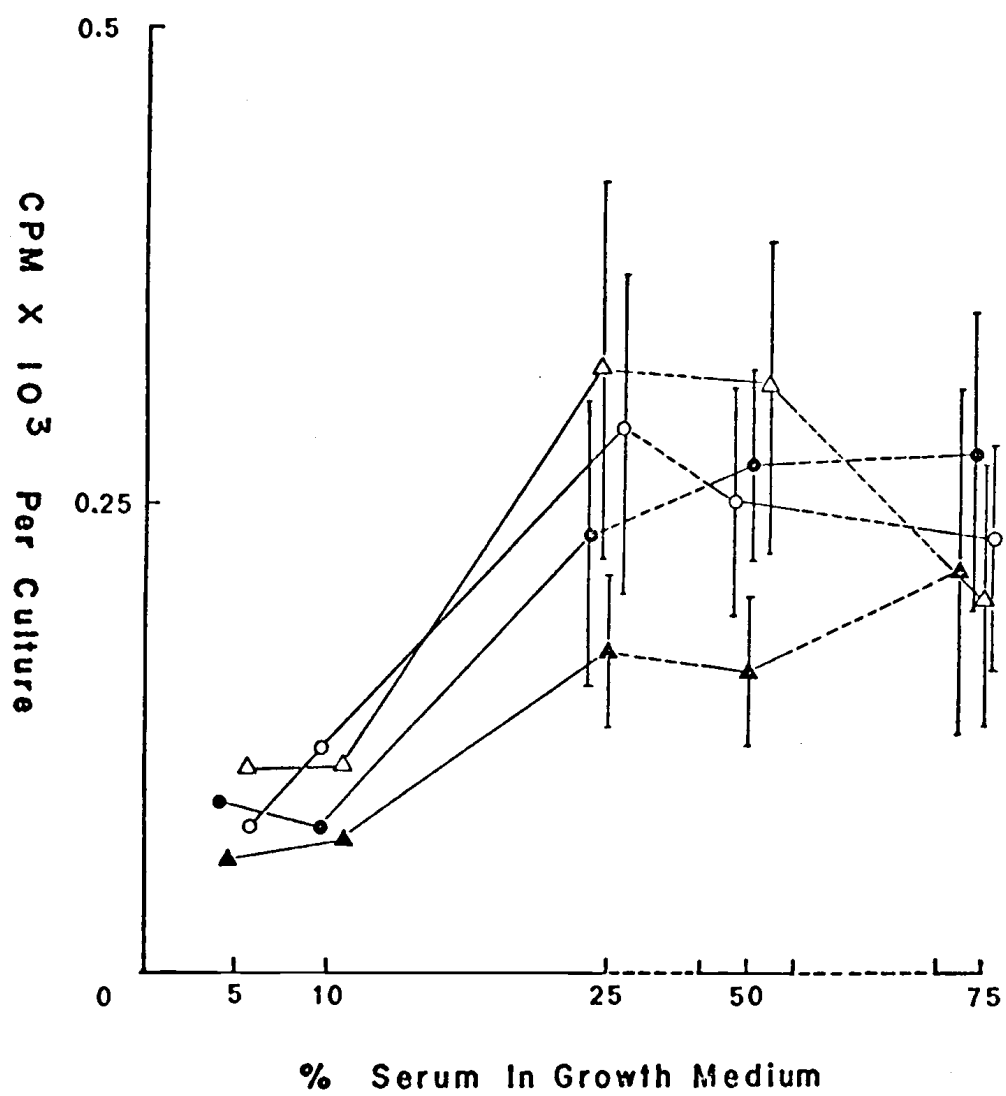Stage I   ●
Stage II   o
Stage III   △
Stage IV   ▲

Figure 12

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}H-thymidine uptake at 2.89×10^3 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}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}H-thymidine incorporation by the PFCP of Stages I and IV. The magnitude of ^{3}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 7.59×10^3 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 $\pm$ SEM.

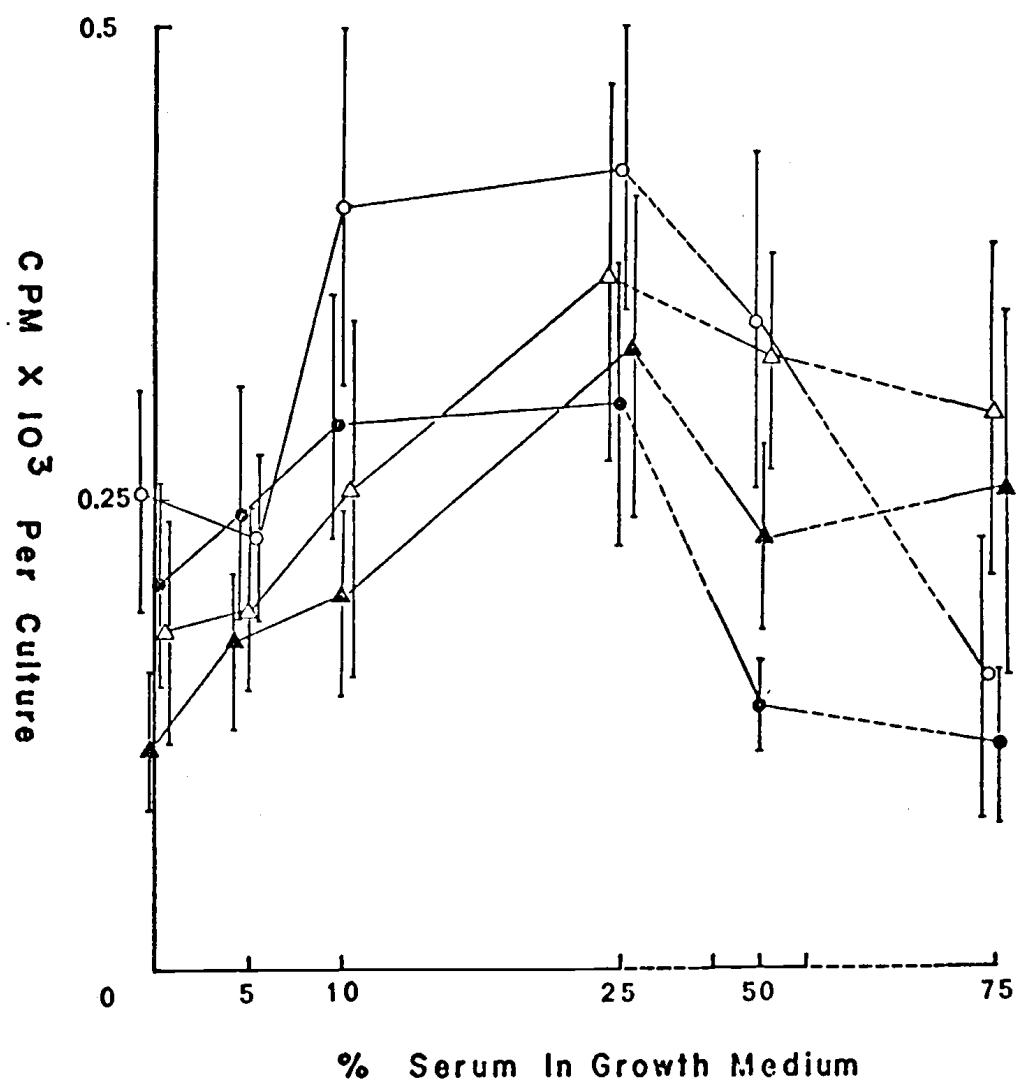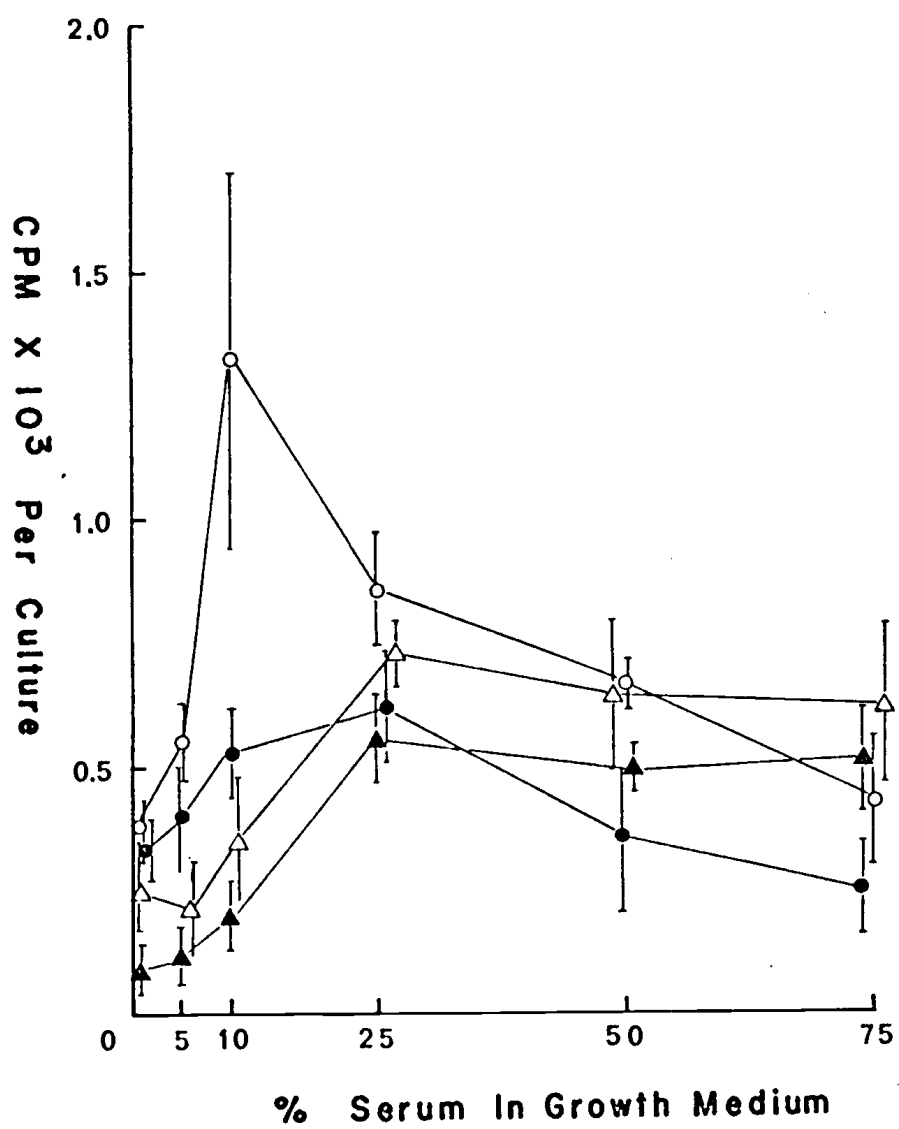Stage I   ●
Stage II   o
Stage III   △
Stage IV   ▲

Figure 13

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 $\pm$ SEM.

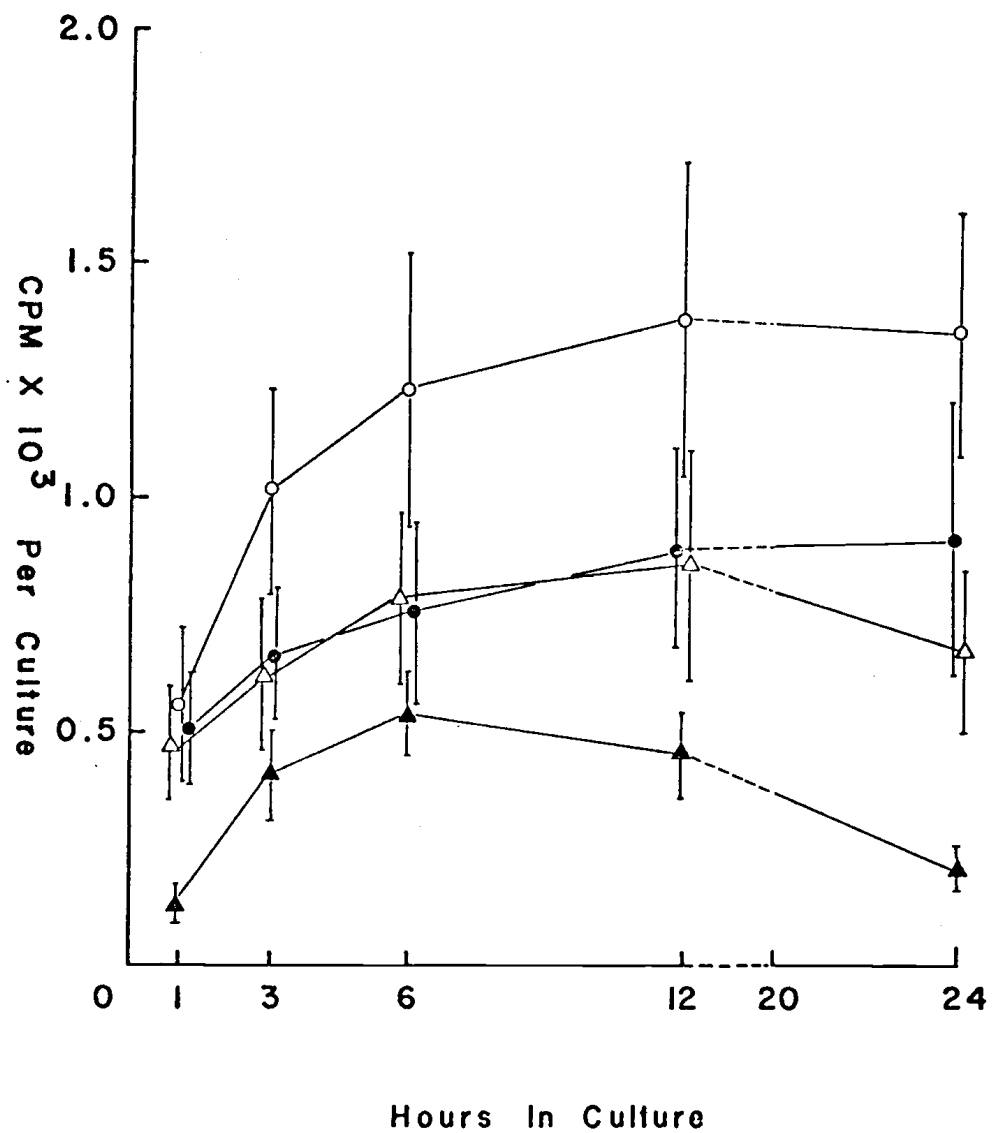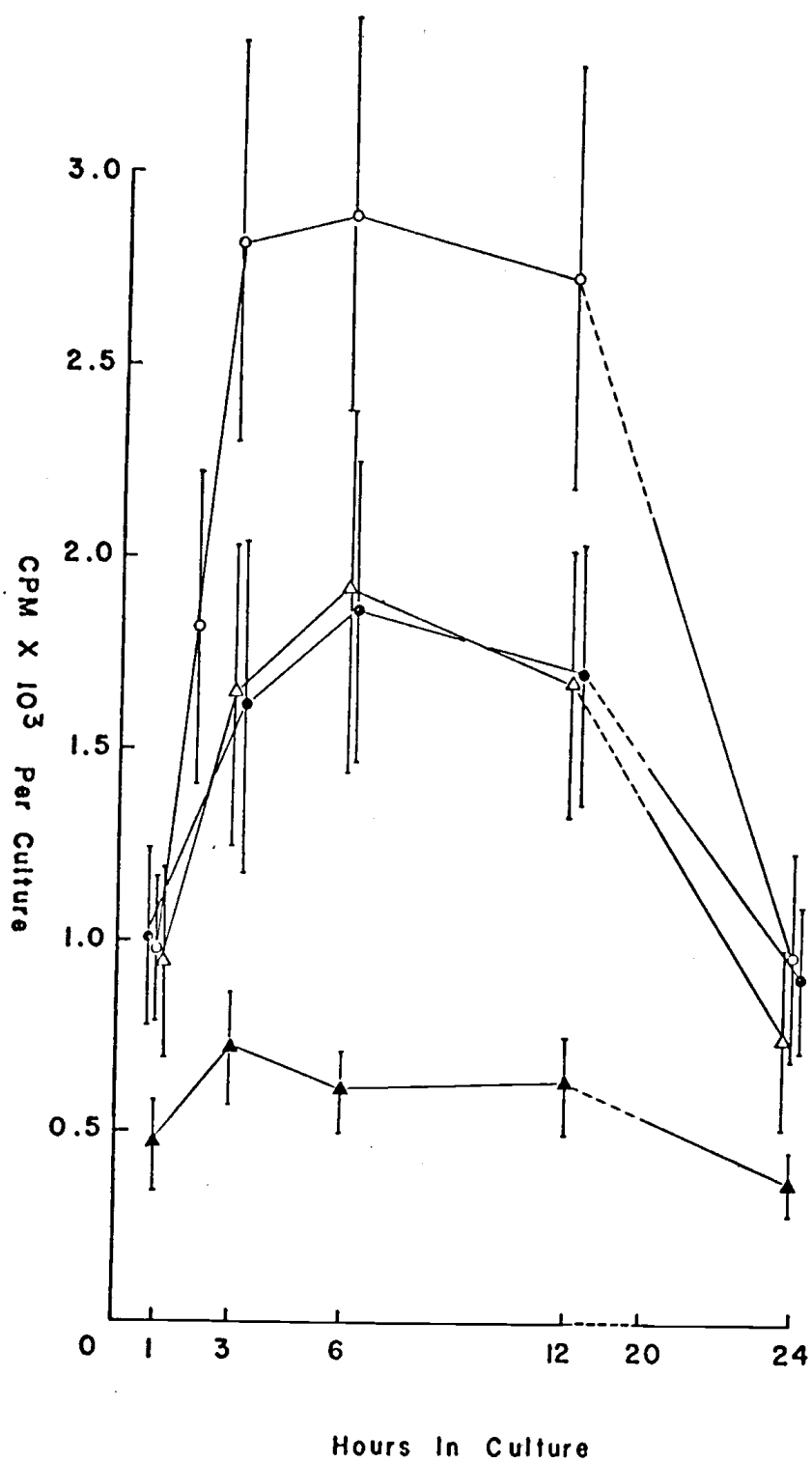Stage I   ●
Stage II   o
Stage III   △
Stage IV   ▲

Figure 14

Hours In Culture

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 2×10^6 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 15.1×10^4 cpm and 13.9×10^4 cpm culture, respectively. Stage I peaked at 11.8×10^4

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 $\pm$ SEM.

             Stage I   ●
             Stage II   o
             Stage III   △
             Stage IV   ▲
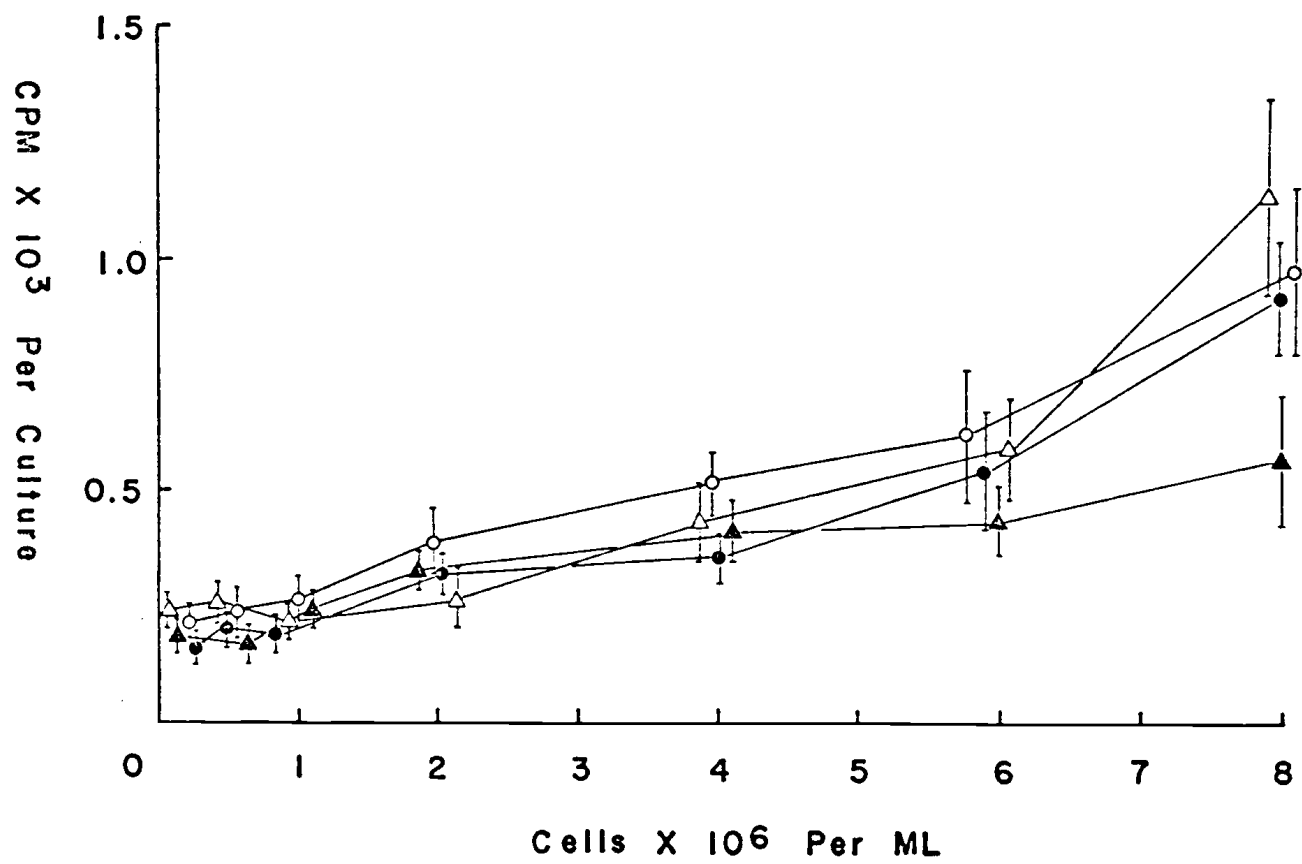
Figure 15

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 $\pm$ SEM.

Stage I   ●
Stage II   o
Stage III   △
Stage IV   ▲

Figure 16
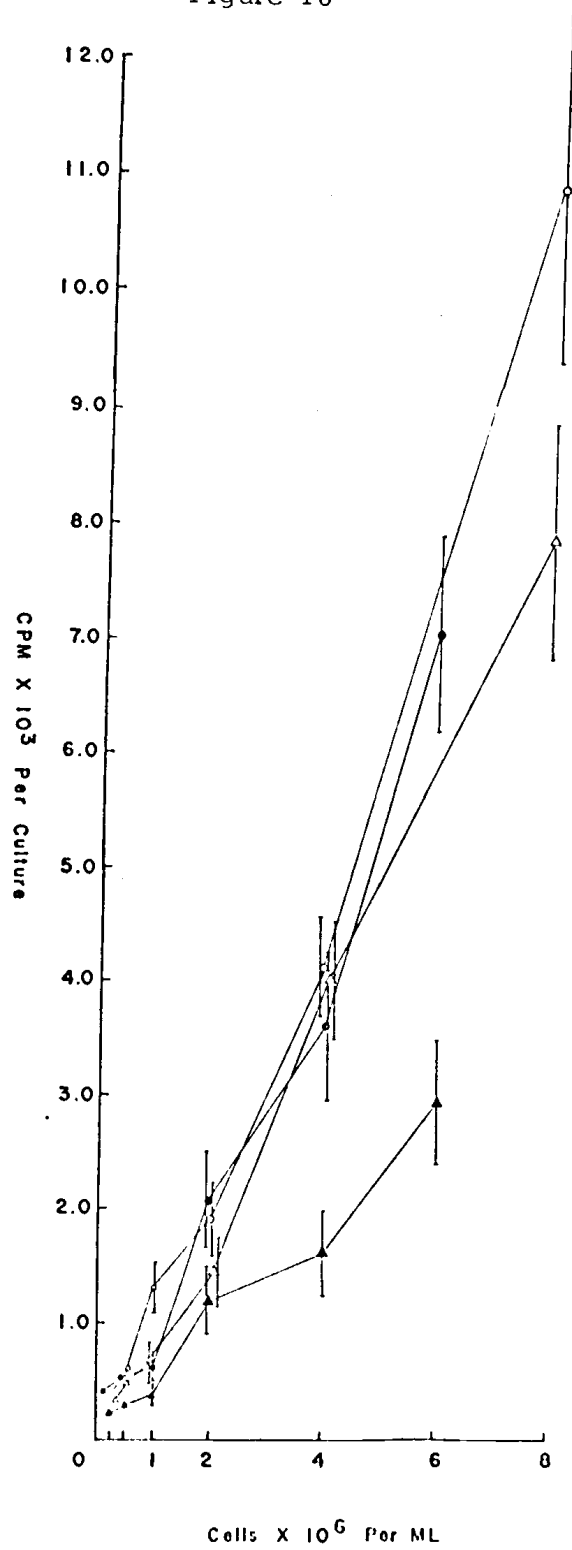
FIGURE 17. Effects of Cell Concentration on Con A
Activated Age-Related PFCP, Expressed
as ^{3}H-Thymidine Incorporation per Culture
in CPM x 10^3. Symbols Represent the
Mean of Three Animals $\pm$ SEM.

Stage I   ●
Stage II   o
Stage III   △
Stage IV   ▲

Figure 17
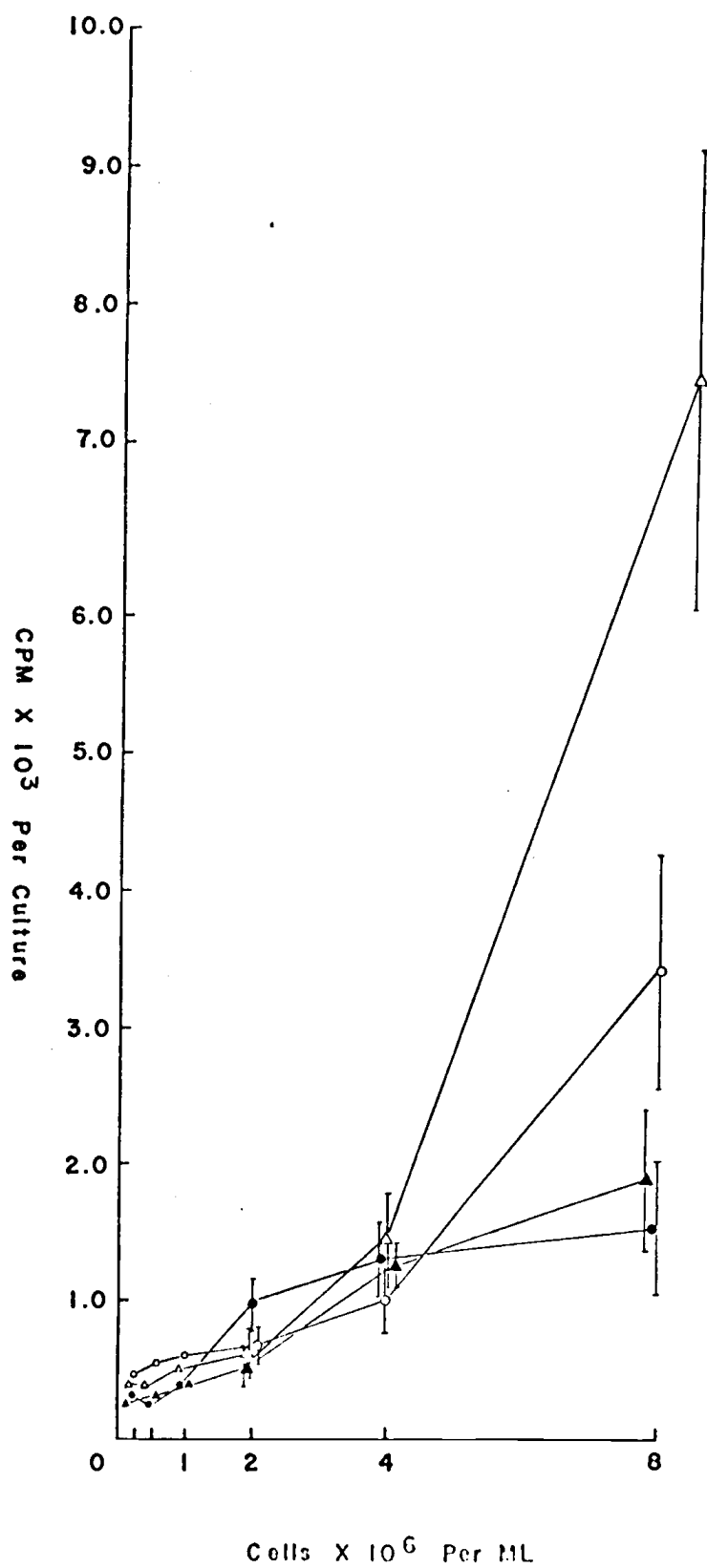
FIGURE 18.   Effects of Cell Concentration on Con A
Activated Age-Related FCP, Expressed as
^{3}H-Thymidine Incorporation per Culture
in CPM x 10^3. Symbols Represent the
Mean of Three Animals $\pm$ SEM.

Stage I   ●
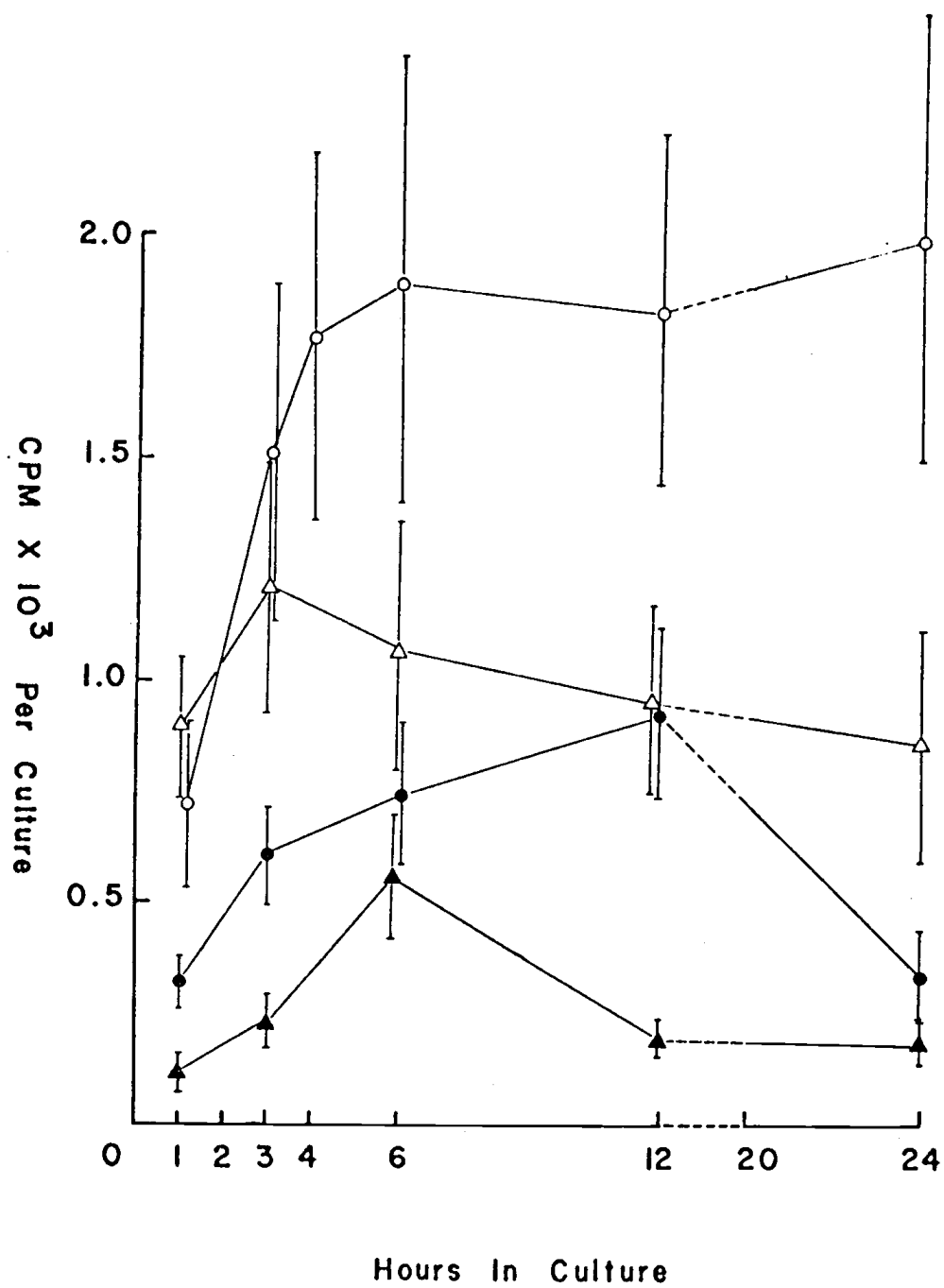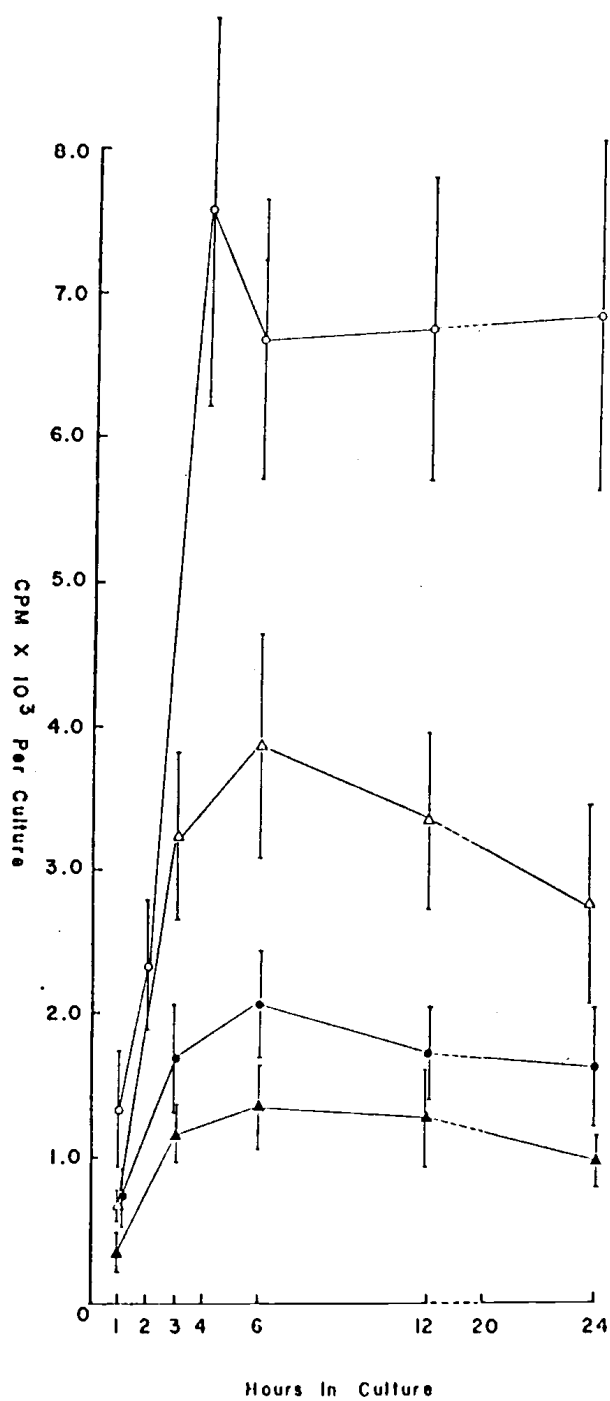Stage II   o
Stage III   △
Stage IV   ▲

Figure 18
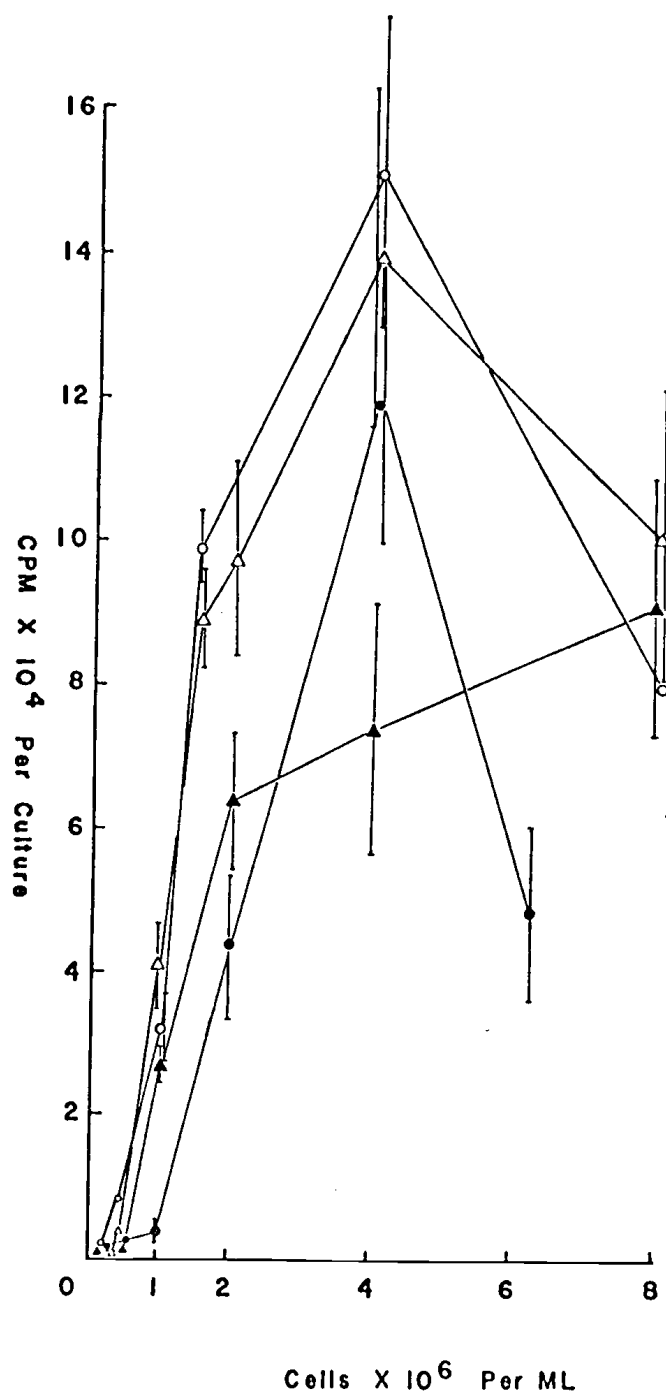
cpm without experiencing a decline in the rate of uptake. Peak incorporation values for Stages II, III and I occurred at cell concentrations of 4×10^6 per ml, and all three stages showed a drastic decline in ^{3}H-thymidine uptake with increases in cell concentration. Stage IV FCP had a sharp break in incorporation rate which occurred at 2×10^6 cells per ml. This new rate was maintained and the final peak uptake value was observed to be 9.1×10^4 cpm at 8×10^6 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 (4×10^6 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)

Figure 19a
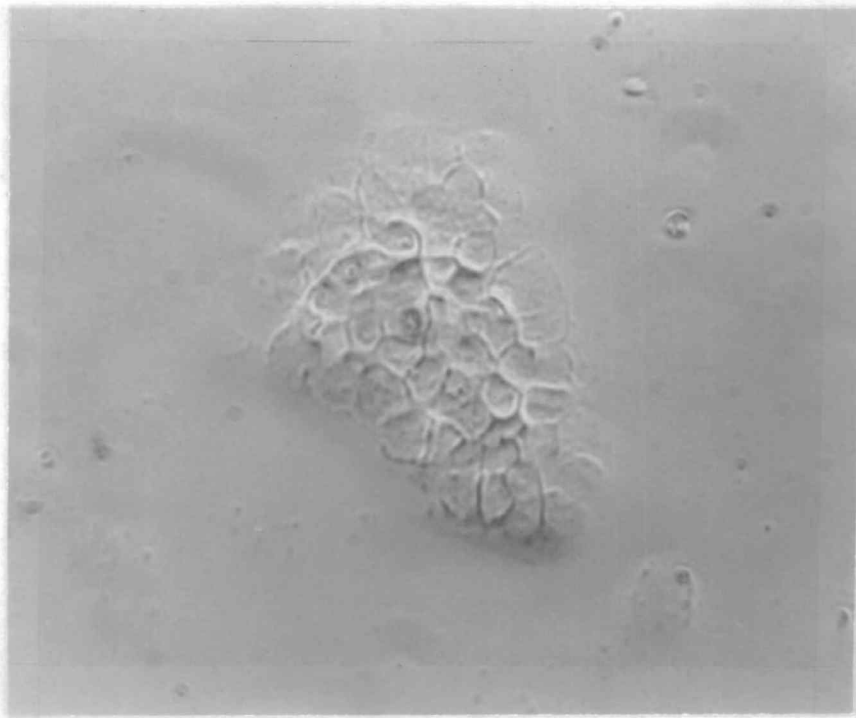
Figure 19b
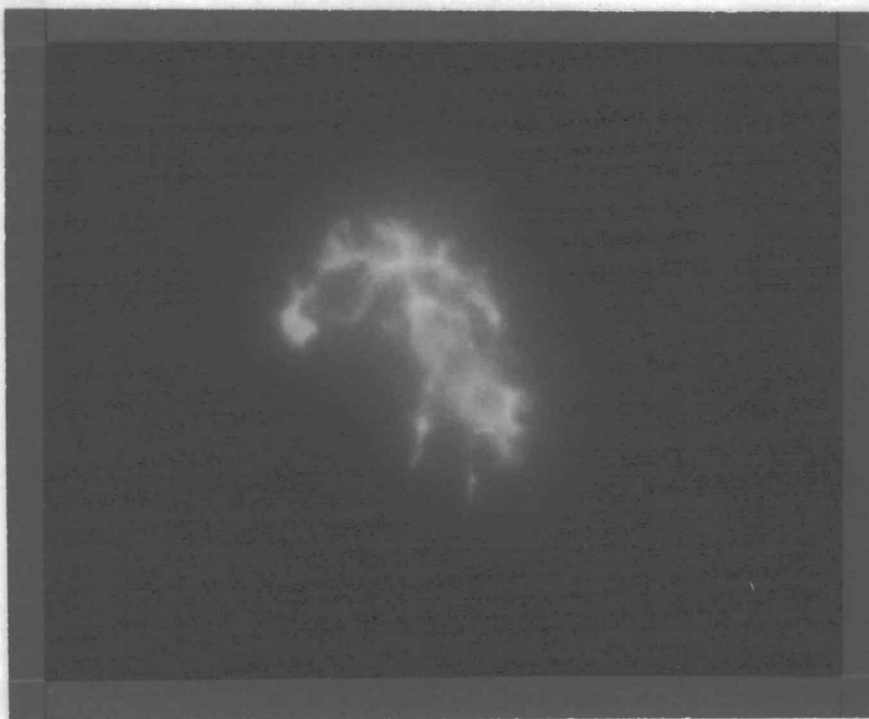
Table II. Effects of Supernatant from Con A Activated 4×10^6 Cells per ml Cultures on Con A Activated 0.5×10^6 Cell per ml Cultures, Expressed as ^{3}H–Thymidine Incorporation in Counts Per Minute (CPM) $\pm$ SEM.

Stage of thymus	Without supernatant	With 10% supernatant	With 50% supernatant
Stage I	550 ± 121	500 ± 95	410 ± 90
Stage II	820 ± 156	770 ± 169	590 ± 100
Stage III	780 ± 172	690 ± 124	510 ± 107
Stage IV	470 ± 94	380 ± 84	260 ± 47

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 cultures 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 incorporation 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 significant 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 compared 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) $\pm$ SEM.

Stage of thymus development	34°C	37°C	40°C
Stage I	590$\pm$106	550$\pm$121	610$\pm$125
Stage II	420$\pm$ 88	820$\pm$156	1300$\pm$210
Stage III	350$\pm$ 74	780$\pm$172	940$\pm$187
Stage IV	230$\pm$ 46	470$\pm$ 94	520$\pm$114

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) $\pm$ SEM.

Stage of thymus development	Incubation time = 64 hrs.	Incubation time = 138 hrs.	Residual Con A Responsiveness $\dfrac{138 \text{ hrs. culture}}{64 \text{ hrs. culture}}$
Stage I	580$\pm$124	280$\pm$51	0.49
Stage II	870$\pm$161	400$\pm$74	0.46
Stage III	810$\pm$182	470$\pm$81	0.58
Stage IV	520$\pm$101	280$\pm$55	0.53

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)

Figure 20a
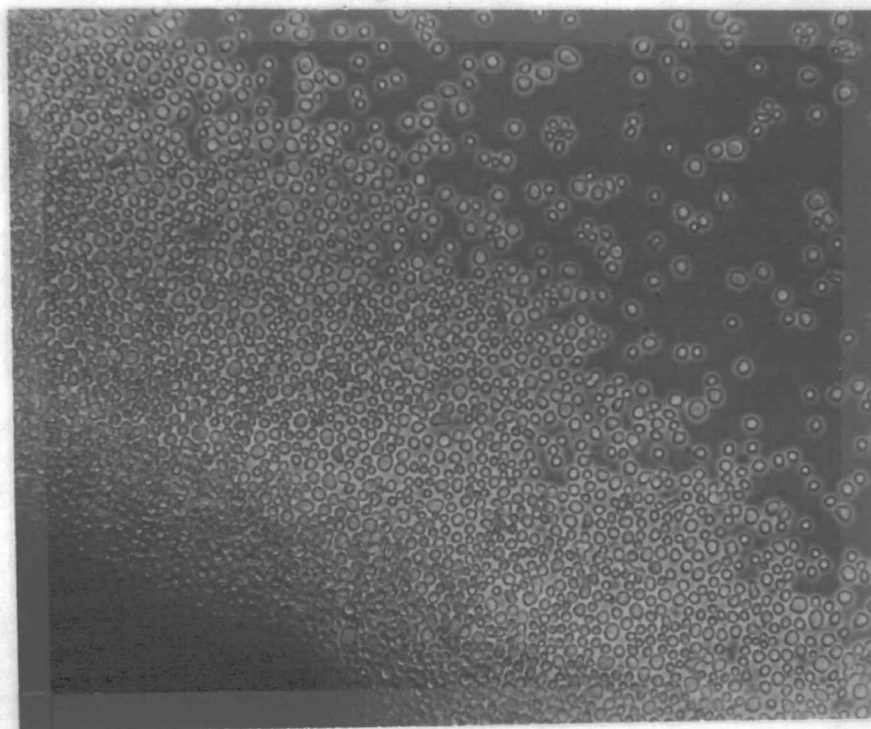
Figure 20b
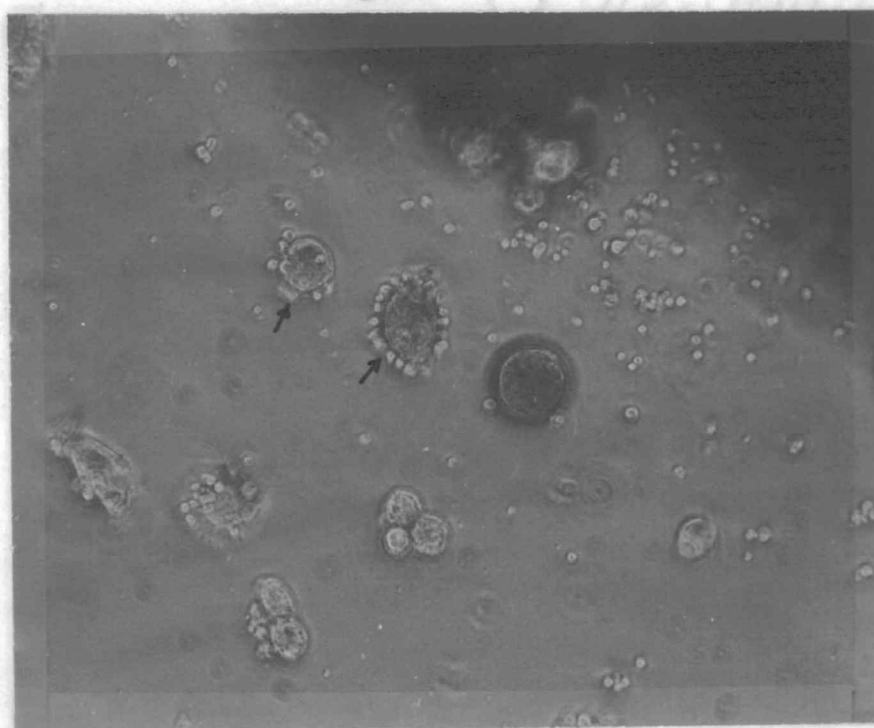
complexes usually disintegrate within 24 hours. Degeneration of TLC was very rapid after the fifth day in culture. Most TLC, degenerated cells and debris were removed by repeated washing with fresh growth medium.

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 progessively 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

FIGURE 21a. A Cell Monolayer Grown from Explants of a
Growing Rat Thymus, Consisting Entirely of
Reticuloepithelial Cells (REC).
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)

Figure 21a
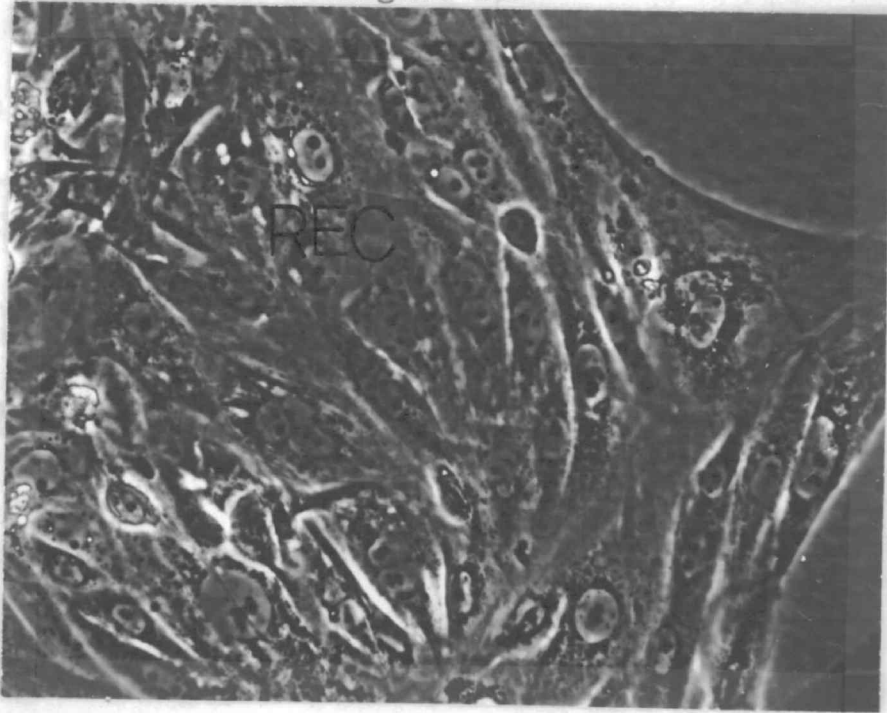
Figure 21b
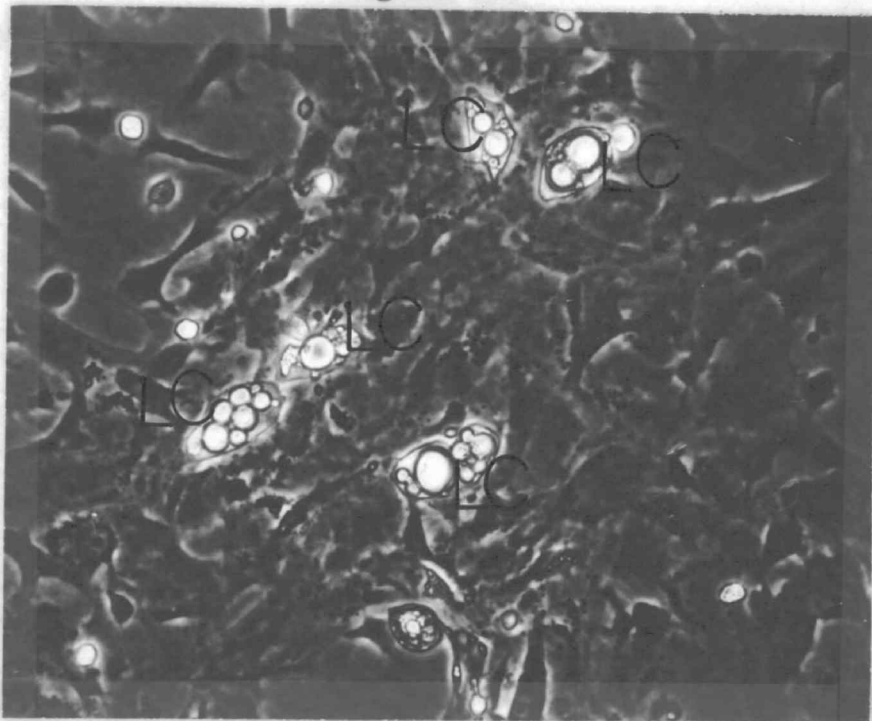
Table V. Differential Cell Counts of Thymic Non-Lymphoid Cells in Explant Cultures,
Expressed as Means of at Least Six Animals ± SEM.

Stage of thymus development	Granular reticulo -epithelial cells (REC)	%	Free macrophages	%	Lipoid Cells	%
Stage I	170±6.7	85	29±6.5	15	0--1	< 1
Stage II	165±8.0	83	32±7.0	16	3±1	1.5
Stage III	161±9.9	81	30±6.0	15	9±3.5	5
Stage IV	112±13.2	56	36±7.8	18	37±6.2	19

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)

Figure 22a
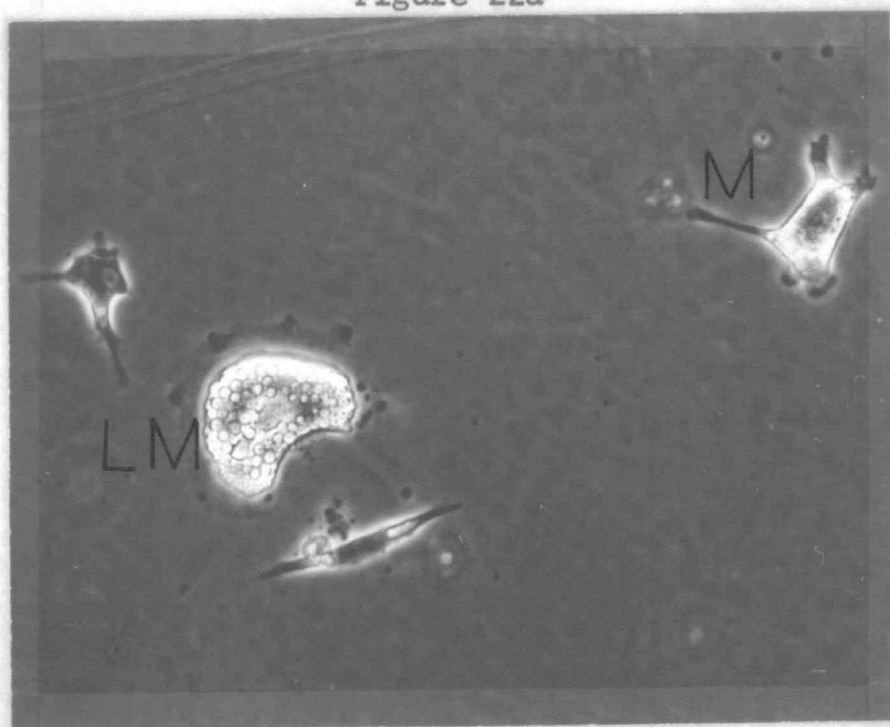
Figure 22b
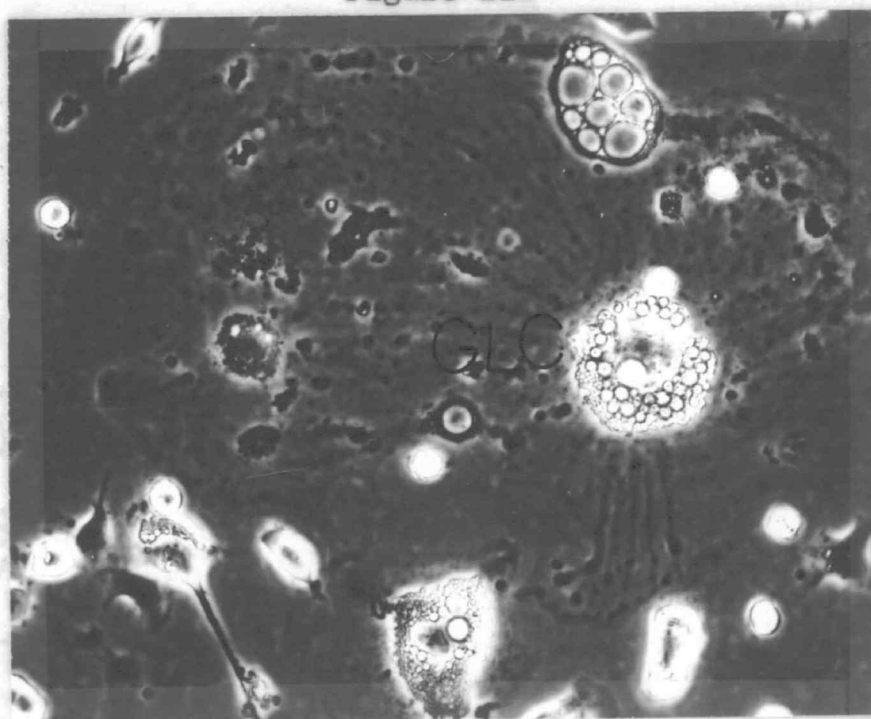
degenerative in culture much faster (within 3 weeks) than their counter-

parts 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 granuoles. 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 aggres-

sive 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 macro-

phage 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.

FIGURE 23. Behavior of a Free Thymic Macrophage in Culture
(Time-Lapse Photomicrography at 44 sec/frame).
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 Macro-
phage (M) (88 min).

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

    e. Dislodgement of Monolayer Cell (C) by Macro-
phage (M) (198 min).

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

    g. Phagocytosis of Dislodged Cell (C) by Macro-
phage (M) (374 min).

    h. Exit of Macrophage from Monolayer (660 min).

Figure 23a
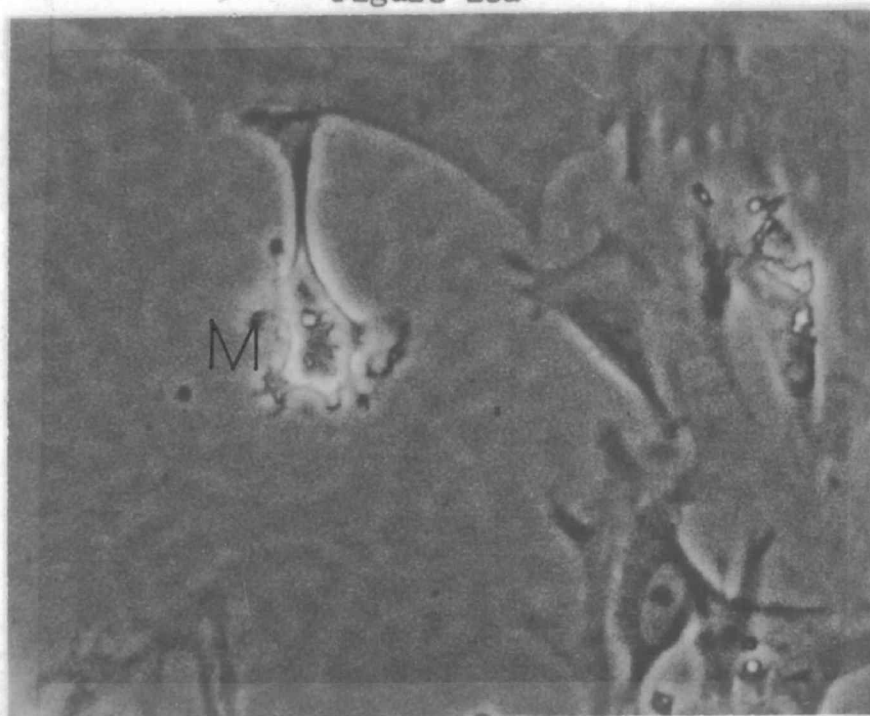
Figure 23b
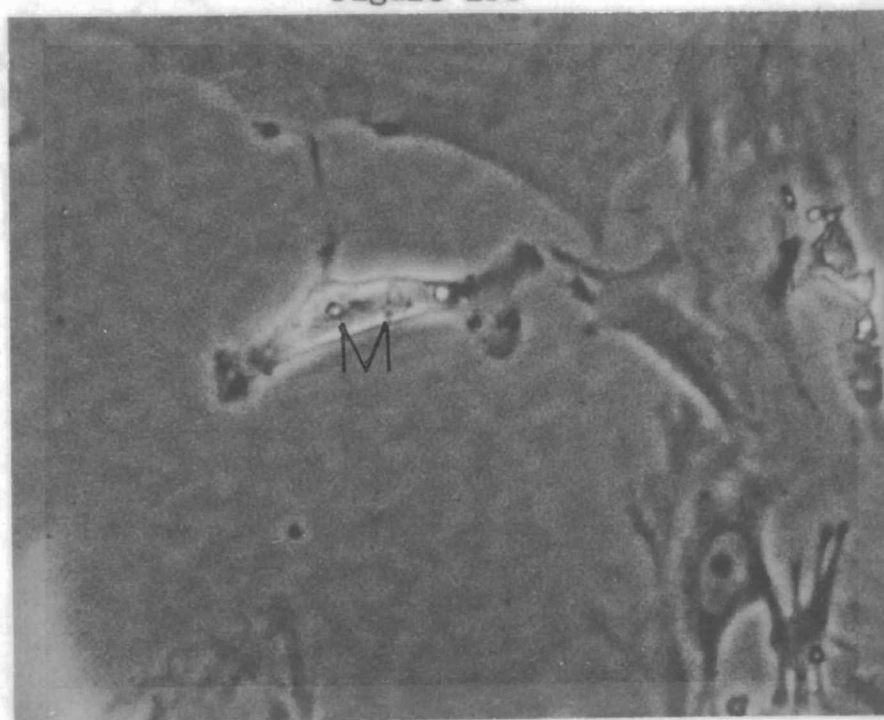
Figure 23c
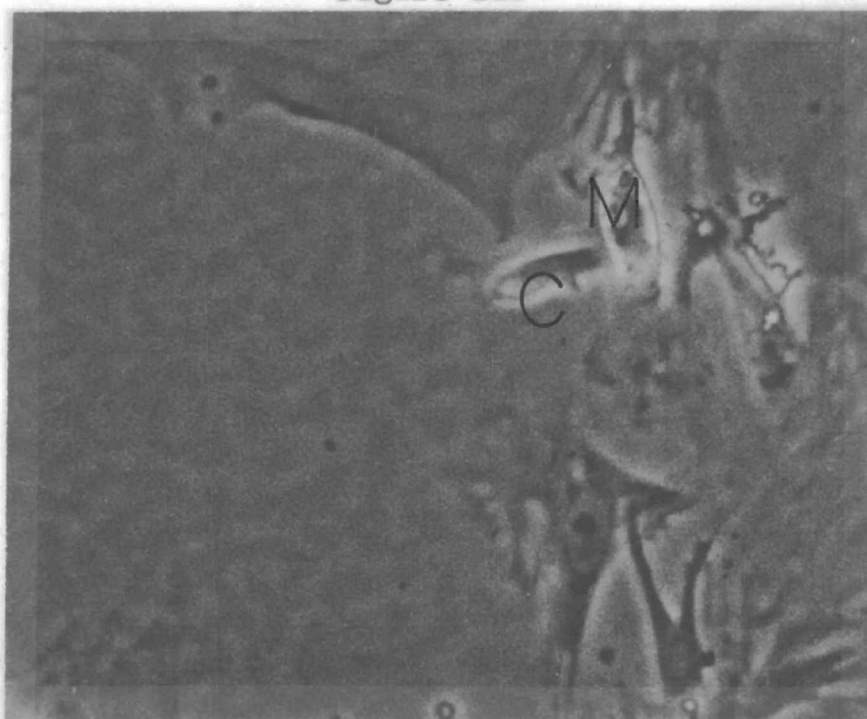
Figure 23d
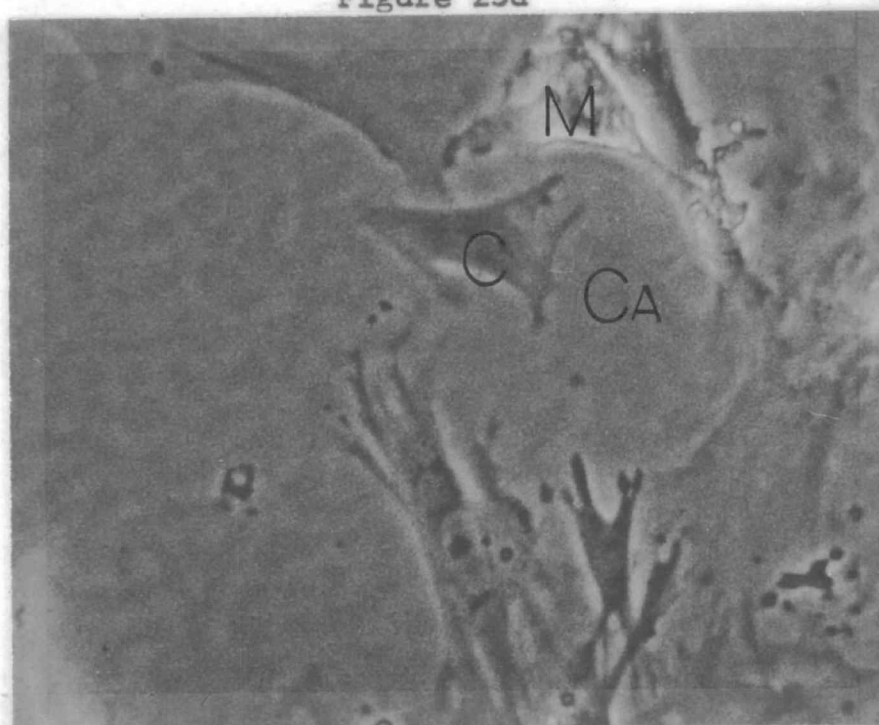
Figure 23e
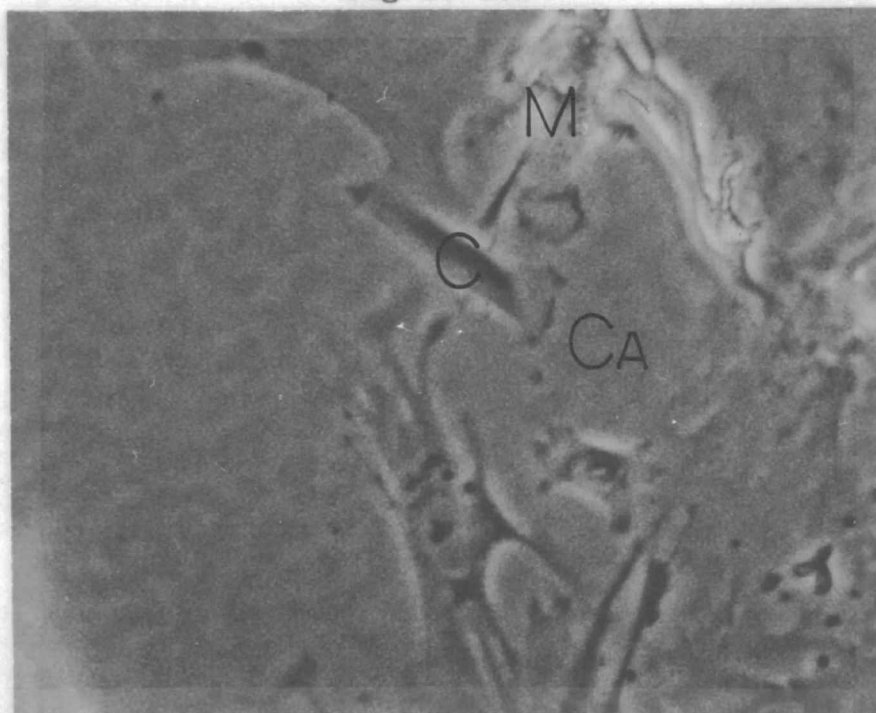
Figure 23f

Figure 23g

Figure 23h
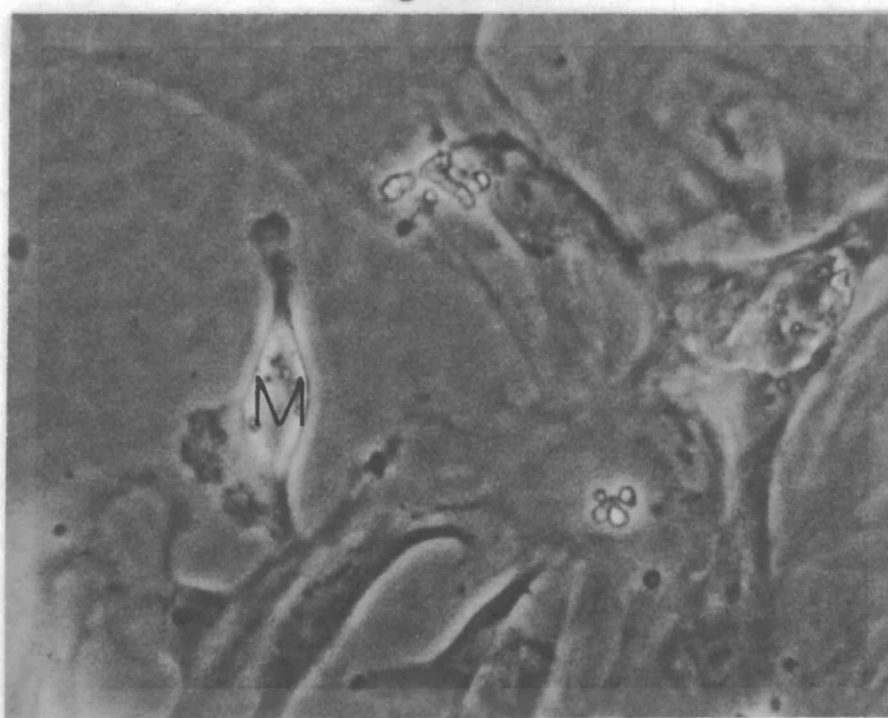
FIGURE 24. A Cell Monolayer Culture from a Stage III Thymus,
Showing: Reticuloepithelial Cells (REC), Lipoid
Cells (LC), and Macrophages (M).
Age of Animal ---- 95 Days.
Age of Culture --- 16 Days.

Figure 24
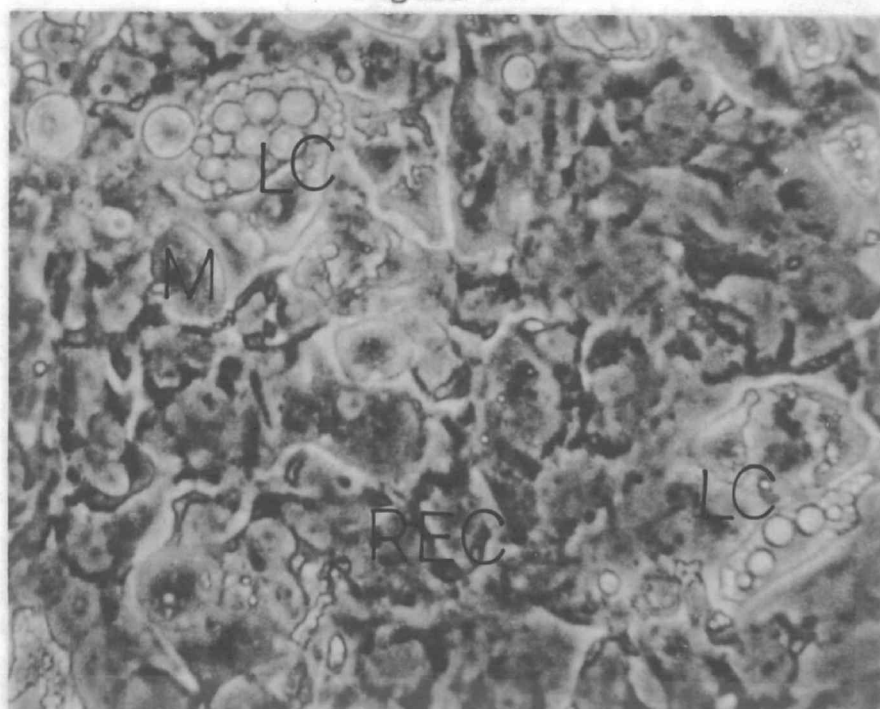
DISCUSSION

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 pre-

dominant 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 $377u^3$, $157u^3$ and $101u^3$, respectively. A study by Zucker et al.

(1976) shows that the thymic lymphoid cell population of the mouse fell

into size classes of $250u^3$, $146u^3$ and $113u^3$. This indicates a similari-

ty 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 re-

sponse of STL to Con A. The more than twofold increase in ^{3}H-thymi-

dine 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.

<u>Comparison of the Effects of Serum Concentration</u>
<u>on Age-Related PFCP and FCP</u>

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 $57^{\circ}C$. 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 consistant 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 ex-

periments 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}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}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 TCP

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 4×10^6 to 6×10^6 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 inter-cellular 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 x 10^6 to 1.5 x 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 thymo-

cyte-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 concentra-

tion 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). Appar-

ently 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 concentra-

tion 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-inhiting 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 $37^{\circ}C$ 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 thymis 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

data. 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 lipoid 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 lipoid 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 lipoid 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.

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