AN INVESTIGATION INTO THE EFFECT OF COMPLETE FREUND'S ADJUVANT

ON THE LONG-LIVED AND SHORT-LIVED LYMPHOCYTES IN THE

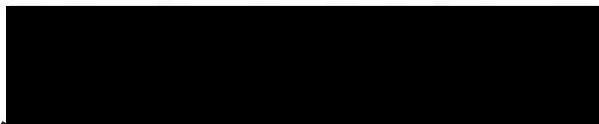PERIPHERAL BLOOD OF MICE


by


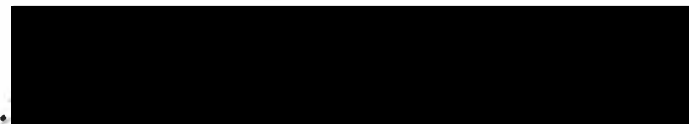Susan Ann Quick Lawrence, B.A.


A THESIS

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# INTRODUCTION

Once considered little more than "phlegmatic spectators" (42), shrouded in mystery, lymphocytes have entered the limelight of experimental investigation during the last 15 years. Making up about 20% of the blood leukocytes in many and variable proportions in other mammals, lymphocytes are not, however, restricted to the blood. Occuring as the major cellular type in the lymph nodes, spleen, thymus and Peyer's patches, lymphocytes are also distributed diffusely through other tissues such as bone marrow and intestinal submucosa.

Lymphocytes are without striking morphological features. They measure about 8μ across in fixed preparations, and have very little cytoplasm. Unlike other leukocytes, they are neither phagocytic nor chemotactic. It would appear that the majority of peripheral lymphocytes at any one moment are not performing any particular function, but are transitional forms between production and function (93). At least some of these functionally resting cells are capable of enlargement into cells which have the characteristic features of lymphoblasts in that they undergo subsequent division.

The capacity of some normal lymphocytes to undergo such transformation and division has been clearly documented by a variety of experimental techniques (75, 39). That portion of the lymphatic system which has the capacity of transformation may be considered as consisting of cells which live a life cycle, rather than having a defined temporal existence such as granulocytes or erythrocytes. Having the appearance of lymphocytes, these may be envisioned as intermitotic cells (18). The time between successive mitoses of such cells may be very long, in terms of months or years. In fact,

the longevity of many of the lymphocytes in the circulating blood
has been established by a variety of techniques employing nuclear
labeling (1, 14, 28, 29, 30, 45, 54, 62, 69, 73, 78). All of these
studies have yielded similar indications of two populations of
lymphocytes: one of these is long-lived in terms of months or years;
the other is short-lived in terms of an average turnover time of days.

Since Everett and his group (27, 28, 29, 30) were among the
initial investigators in this field as well as the fact that their's
was the most extensive study using tritiated thymidine in relation
to long-lived and short-lived lymphocytes, a cursory review of their
findings is presented. Tritiated thymidine was administered in
single or multiple injections, or by continuous infusion. The find-
ings were based on analysis of autoradiographic smears from the
thoracic duct lymph, lymph nodes, peripheral blood, spleen, thymus,
and bone marrow in young rats. Everett, et al. (28) calculated that
approximately 10% of the thoracic duct lymph, 25% of the lymph nodes,
35% of the peripheral blood, 70% of the spleen, 95% of the thymus,
and 100% of the bone marrow lymphocytes were short-lived.

Although autoradiography, at present, offers the best method
for studying long-lived and short-lived lymphocytes, it must be rec-
ognized that certain controversial assumptions and premises are
necessary for the analysis of tritiated thymidine studies. The
following were listed by Cronkite, et al. (19) and Everett, et al. (27).

1. The radioelement has the same chemical reaction with the
   cell as does the normally occuring element.

2.  Tritium labeled thymidine initially is uniformly distribut-
    ed throughout the body and is either promptly incorporated
    into DNA or degraded.

3.  The tritium and labeled thymidine are largely nonexchange-
    able after DNA is tagged.

4.  DNA turnover is due solely to cell division and death.

5.  Reutilization of labeled material is insignificant.

6.  DNA synthesis in general destines a cell to divide again.

7.  In vitro labeling determines the proliferative potentials
    of the cells.

8.  In vivo labeling makes possible the study of the kinetics
    of cell proliferation.

9.  There is no disturbance of the normal cell generative cycle
    by radiation injury from the tritiated thymidine incorporat-
    ed into DNA or from the formation of labeled degradation
    products.

The credibility of isotope labeling, despite the many assump-
tions necessary, has been enhanced by the recent studies using mor-
phological chromosome markers. This method is based on the fact
that radiation produces acentric chromosome fragments in the lympho-
cytes. Such chromosome fragments are almost never found except in
the cells of people who have been exposed to radiation. Moreover,
since acentric fragments lack centromeres that control chromosome
movement on the mitotic spindle, they will not be distributed by
the spindle to the daughter cells; instead they will be lost to the
nuclei and degenerate in the cytoplasm (84) at the first cell

division after radiation. Therefore, an acentric chromosome fragment in the lymphocyte is assumed to mean that the lymphocyte has not divided between the time the patient was exposed to radiation and the time of observation; and the rate of decrease of lymphocytes with acentric fragments provides a direct measure of the lifetime of the lymphocyte.

Studies using the above morphological chromosome marker method support the hypothesis of a long-lived population of lymphocytes (4, 5, 10, 66, 68). Recently Norman and his associates (66) investigated the blood lymphocytes of hematologically normal women at intervals following curative local irradiation for carcinoma of the cervix. Some of the lymphocytes which passed through the irradiation field during the time of exposure incurred nuclear chromosome injuries which could be detected by karyotyping the cells in metaphase. By scoring those chromosome injuries, which would have been lost had the cell undergone a division prior to the time of the induced mitosis, these workers showed that the average life of the lymphocyte was 530 days. The study demonstrated that lymphocytes with the latent capacity to transform may exist for very long periods without dividing and are recirculating cells, since all samples of analysis were taken from the circulating blood at intervals following local irradiation to the pelvic region.

In a similar experiment Buckton and Pike (10) found lymphocytes with chromosome injuries in blood samples cultured up to 5 years after radiation exposure. Apparently these cells were in their first division since their exposure to radiation. Nowell, (68), using similar patient

material and techniques, demonstrated that, in tuberculin-sensitive patients receiving local radiation therapy, the long-lived lymphocytes which incurred the chromosome injury transformed in response to the tuberculin protein. Nowell (91) considers that his findings lend credence to Fitzgerald's theory (34) that lymphocytes specifically "committed" to an antigen are present in the peripheral blood of normal humans; that these cells may survive in the body for years without dividing, and that they are still capable of responding by proliferation when re-exposed to the sensitizing agent. Therefore, long-lived, non-dividing, "committed" lymphocytes may be responsible for long term immunity (34,41).

The distribution of the two lymphocyte populations has been well documented for the rat (14, 16, 28, 29). While long-lived lymphocytes are predominant in thoracic duct lymph and are abundant in the lymph nodes, lymphocytes produced in the thymus, bone marrow and germinal centers are of the short-lived variety.

A functional role for the short-lived lymphocyte has been the subject of much speculation but relatively inconclusive experimental evidence. It has been suggested that the short-lived lymphocyte is perhaps more concerned in the processing of antigen which precedes immunocyte proliferation than in providing cells which express the immune response by producing antibody (18, 67). In support of this hypothesis is the fact that moderate irradiation injury and cortisol administration, both of which seriously deplete the animal of short-lived lymphocytes, impair the immune response by disrupting the antigen-processing phase of the reaction (17). One might assume

that the macrophage is responsible for antigen processing and is the cell destroyed by irradiation and cortisol. However, this is not the case because neither of these treatments depresses macrophage production (17, 88, 89).

Everett and Tyler proposed, "...that the extensive proliferation of short-lived lymphocytes in the bone marrow, thymus, and germinal centers is necessary for producing tolerance to auto antigens" (31). Extensive proliferation, it has been stated, is necessary to code or type antigens; consequently, a few short-lived cells may be selected for long life span (61). A further function of short-lived lymphocytes is that of serving as trephocytes (28). This is in keeping with the supposition that lymphocytes may exert a nutritional or "trophic" function (15, 32, 55). According to Gowan, "...it has been suggested that it is the withdrawal of this [trephocytic] influence which leads to wasting diseases" (40). Physical characteristics of wasting disease, ruffled fur, hunched posture, diarrhea, weight loss, and atrophy of the thymus were apparent in Morton and Siegels study, Hematological Changes in Mice Following Freund's Adjuvant Administration (64). Morton and Siegel also observed a diminution in peripheral blood lymphocytes that lasted for approximately 33 days. Consequently, it seemed of some interest and importance to investigate whether this long- or short-lived lymphocyte population was preferentially being depleted from the peripheral blood as a result of a single intraperitoneal injection of complete Freund's adjuvant.

Freund introduced adjuvants consisting of mineral oil, water and tubercle bacilli, emulsified with lanolin (35). This complete Freund's adjuvant is commonly used for enhancing and prolonging antibody response to certain antigens. The basic mechanism of action of complete Freund's adjuvant remains obscure.

## MATERIALS AND METHODS

### Experimental Design

Twenty mice were divided into three groups. Groups I and II consisted of eight mice each, and group III consisted of four mice. Groups I and II received the course of tritiated thymidine injections described below. Groups II and III received the single injection of complete Freund's adjuvant forty hours after the first injection of tritiated thymidine. Four mice from Groups I and II, and two mice from Group III were sacrificed twelve hours after the last injection of tritiated thymidine. The remaining ten mice were sacrificed five days later. Blood values and weights were obtained on the first, third, fifth, seventh, ninth, eleventh, and sixteenth days post adjuvant.

Group III was included in the experimental design to gauge the effect, if any, that frequent injections of tritiated thymidine had on the complete Freund's adjuvant injection. The results obtained on the Group III animals parallel those of the Group II animals. Therefore, they are not included in the results listed below. Henceforth, the Group I animals shall be referred to as the control animals, and Group II shall be referred to as the experimental animals.

### Mouse Stock

Seven-week-old female BALB/c mice, weighing sixteen to twenty-two grams, were obtained from the Jackson Laboratory, Bar Harbor, Maine.

## Freund's Adjuvant

Twelve mice received a single intraperitoneal injection of 0.25 ml of a mixture composed of Freund's incomplete adjuvant (Difco) with 1 mg of M butyricum (Difco) emulsified with saline.

## Tritiated Thymidine Labeling

Sixteen mice were given tritiated thymidine (thymidine-methyl-H3, Schwarz Bioresearch, Inc., Orangeburg, New York) specific activity 1.9 c per mmole (0.5 uc per gram body weight) by intraperitoneal injection at twelve-hour intervals (6:00AM, 6:00PM) for 12½ days (25 injections). While continuous infusion is, perhaps, the ideal method for administering tritiated thymidine, it is not readily nor conviently achieved; whereas injections every 12 hours have produced similar labeling curves (19, 54, 78).

## Preparation of Autoradiographs

Blood smears were obtained by orbital bleeding at 7:00 o'clock each morning. The mice were sacrificed by cervical fracture. Bone marrow smears were obtained from the femora and imprints made of the thymus, spleen and lymph nodes.

The blood and marrow smears, and the tissue imprints were fixed in absolute methyl alcohol prior to dipping in NTB-3 emulsion (Eastman Kodak). Low intensity light with Wratten series A filter was used sparingly at a distance of three feet. This emulsion was melted in a water bath at 42° C. After 3 weeks exposure at 4° C, the slides were developed for two minutes in Dektol, rinsed in tap

water, fixed in Kodak acid fixer for ten minutes and rinsed for twenty minutes in running tap water. The autoradiographs were then stained with hematoxylin.

## Analysis of Autoradiographs

A minimum of three smears from each mouse bleeding, and two preparations from each tissue were examined. A minimum of one-hundred lymphocytes from each of three slides (totaling three hundred) were examined to determine the percentage and intensity of labeling in the peripheral blood (Plate I). A one-thousand cell differential was made on the bone marrows, and those lymphocytes encountered in this differential were used in the analysis of lymphocyte labeling in the marrow. Five hundred lymphocytes were counted on the imprint slides of the thymus, spleen and lymph nodes. Cells with more than 3 grains over the nucleus were counted as labeled.

## Histology

Portions of the thymus, spleen, lymph nodes, liver and kidney taken for histological examination were fixed in Bouin's fluid, and after paraffin embedding were cut at $6\mu$. Sections were stained with hematoxylin and eosin.

## White Blood Cell Counts and Volume of Packed Red Cells

These were done by standard methods. The blood cells were suspended in 0.85% NaCl and counted on a Model A Coulter Counter (11).

The volume of packed red cells was determined by the micromethod, using the International microcapillary centrifuge, Model MB and the International microcapillary Reader (82).

Evaluation of Runt Disease

Mice were evaluated on the basis of weight changes and clinical appearance.

## RESULTS

Weight Loss and Clinical Appearance

The mice receiving the injection of complete Freund's adjuvant displayed weight loss, diarrhea, ruffled fur and hunched appearance by twenty-four hours post injection (Plate II). These symptoms persisted for eight days. The weights did not approach normal until after the eleventh day (Plate III). However, one mouse was found dead on the tenth day post adjuvant.

Peripheral Blood

Throughout the experiment, the control animals maintained an average hematocrit of $48.6 \pm 3.0$, in contrast to the experimental group which showed a steady decline to a mean of 37.6 by day eleven (Plate III). The neutrophils remained constant in the control group; but in the experimental group, after an initial fall, the neutrophils climbed to a high of 7,000/cu. mm. (Plate III). The absolute lymphocyte count showed some unexplained fluctuations in the control group, but remained lower in the experimental group (Plate III). The lymphopenia was most severe at twenty-four hours post adjuvant. No change in eosinophil or monocyte numbers was observed, nor were abnormal or immature cell forms seen.

The labeling pattern of the lymphocytes in the peripheral blood of the mice which had received Freund's adjuvant clearly differed from that of the controls (Plate IV). In the control group

the percentage of labeled lymphocytes rose steeply between the first and fifth days; but in the mice which received adjuvant, there was no rise in labeling until the fifth day. The greatest differences between the two groups were noted on the third, fifth, and seventh days. The experimental group was significantly lower on these dates as analyzed statistically by Student's t test ($p < 0.01$). The percentages and absolute numbers of labeled and unlabeled lymphocytes in the control and experimental groups is given in Table I. The transition point from a rapid to a gradual increase in the percent of labeled cells has been considered to be the time when labeling of short-lived lymphocytes was almost complete. The subsequent labeling has been regarded as being due to long-lived lymphocytes entering the circulation. Therefore, from day five post adjuvant ($7\frac{1}{2}$ days of tritiated thymidine injections) to day eleven post adjuvant (12 hours after the last injection of tritiated thymidine) the labeled lymphocytes were considered to be short-lived lymphocytes and the unlabeled lymphocytes were considered to be long-lived lymphocytes. In the control animals, 21% of the lymphocytes on day five, 23% on day seven, 27% on day nine, and 25% on day eleven were short-lived. In comparison, after the animals had received complete Freund's adjuvant, 8% of the lymphocytes on day five, 13% on day seven, 21% on day nine and 22% on day eleven were short-lived. When the absolute numbers of short-lived and long-lived lymphocytes were calculated from the total lymphocyte count, it became apparent that, while the most consistent effect of the adjuvant had been to reduce the number of short-lived lymphocytes, there was also a fall in the numbers of

long-lived lymphocytes after day seven post adjuvant. Although the
total lymphocyte count of the experimental group almost reached
that of the control group on days five and seven post adjuvant, the
adjuvant treated mice were deficient in circulating short-lived
lymphocytes. Following the cessation of tritiated thymidine injec-
tions, the control mice evidenced a steady decline of labeling. The
experimental mice, on the other hand, rose slightly prior to a sharp-
er descent.

## Bone Marrow

The bone marrow evaluation was consistent with the findings in
the peripheral blood. Examination of the bone marrow from the control
and experimental mice revealed that the latter manifested a higher
degree of cellularity.

A comparison of the blast cells in the red cell series showed
the control and experimental groups to be approximately the same on
the eleventh day (Table 2). However, by the sixteenth day, the ex-
perimental group showed a marked reduction. The rubricytes, or older
nucleated red cells, evidenced a percentage approximately equal to
half the control values on both days eleven and sixteen post-adjuvant.
In the control mice, the erythropoietic cells comprised 40.3 per-
cent on day eleven and 37.1 percent on day sixteen; whereas, the total
percentage of erythropoietic cells in the marrow of the experimental
mice decreased from 26.3 percent on day eleven to 17.6 percent on
day sixteen.

In contrast to the control group, the percentage of earlier cell forms in the granulocytic cell series was slightly higher in both experimental counts; those sacrificed at eleven days and sixteen days post-adjuvant. The more mature granulocytes in the experimental mice were slightly higher on the eleventh day, and increased greatly by the sixteenth day. The tabulated eosinophils (Table 2) were noted to be relatively lower in the experimental animals. The total percentage of granulocytes in the marrow of the control group was 48.5 on day eleven and 79.4 percent on day sixteen in the experimental group.

A dissimilarity between the two groups was evidenced by a reduction of the lymphocytes in the experimental mice. No apparent differences were observed between the control and experimental blast and miscellaneous cells.

No difference between the control and experimental groups was observed in the labeling of the lymphocytes in the bone marrow (Table 4). All lymphocytes encountered eleven days post-adjuvant, twelve hours after the last injection of thymidine, were labeled, while all lymphocytes seen in the marrow smears sixteen days post-adjuvant were unlabeled.

Gross and Microscopic Pathology

Gross observations on autopsy revealed severe adhesions between the body and viscera in the affected animals. Two of the experimental animals sacrificed at 16 days post-adjuvant manifested ascites. A smear of the concentrated ascites fluid revealed numerous segmented

neutrophils, lymphocytes, plasma cells, and undifferentiated blast
cells. Tissues collected from the control animals appeared normal
in both early and late examinations.

A significant deviation from the thymus weights of the control
group was noted in the experimental group (Table 3). At eleven
days post-adjuvant they were less than one percent of control values
and increased weight slightly by sixteen days post-adjuvant. Histo-
logically the experimental thymus displayed an increase in reticulo-
endothelial cells and multinucleated giant cells, but was not re-
markable in its loss of lymphocytes from the cortex. At the time
of the early sacrifice, typical cortical tissue consisting of crowd-
ed lymphocytes appeared as a thin band displaced centrally, so that
reticular medullary-like tissue appeared as a subcapsular border as
well as occuring centrally in the lobule (Plates V and VI). By the
time of the late sacrifice, cortical and medullary zones could not
be delineated. Very few cells could be identified as lymphocytes,
most of the cells being reticulum cells.

On the eleventh and sixteenth days post-adjuvant, the spleen
weights manifested a high degree of variance between the control
and experimental groups (Table 3). While the control weights had
decreased slightly, the experimental weights had increased to nearly
twice that of the control on day eleven, and to three and one half
times that of the control on day sixteen. The general architectural
pattern of the spleen was usually preserved. By eleven days post-
adjuvant, the spleen showed signs of extramedullary hematopoiesis
with megakaryocytes and cells of the granulocytic cell series being

numerous in the red pulp (Plate VII). The white pulp contained many mitotic figures. There was hyperplasia of the sinus lining histiocytes and multinucleated giant cells. These changes were more pronounced at sixteen days post-adjuvant.

Lumbar nodes were examined from both control and experimental mice. Although weights were not obtained for lymph nodes, on visual examination marked differences between the two groups was not apparent. The histological picture of the nodes could best be described as a non-caseating granulomatous lymphadenitis resembling Boeck's sarcoid (Plate VIII). The sinuses were distended with neutrophils and macrophages. Cortico-lymphocytosis and medullary plasmacytopoiesis were evident.

In addition to the alterations in lymphoid tissues, increased liver weights were observed (Table 3), which were attributible to Kupfer cell proliferation and peripheral blood laden sinusoids (Plate IX).

An examination of the kidneys, revealed no convincing differences between the experimental and control groups.

Lymphocyte Labeling in Tissue Imprints

The majority of the lymphocytes in the thymus imprints of the control mice, which were examined twelve hours after the course of injections, were labeled (Table 4). However, the thymus imprints of the experimental mice, at the same time, had only 21 percent of the lymphocytes labeled. In the control and experimental imprints, the percentage of lymphocytes still labeled 5½ days after the last injection was much reduced; thus, indicating that the majority of

the lymphocytes in the thymus were of the short-lived variety, and that the lymphocytes lost from the thymus in the adjuvant-treated mice were also short-lived lymphocytes.

A lower percentage of spleen and lymph node lymphocytes were labeled in the control group than in the experimental group at the time of the early sacrifice (Table 4). Although both groups showed a decline in the percentage of lymphocytes labeled by the time of the late sacrifice, the control mice retained a higher percentage of labeled lymphocytes. This indicated that a higher proportion of the spleen and lymph node lymphocytes were long-lived cells, and that the majority of the lymphocytes in the experimental mice were of the short-lived variety. Since histologically, the spleen and lymph nodes of the experimental animals were actively producing lymphocytes, the absolute number of labeled cells may have been the same in control and experimental animals by the time of the late sacrifice; in which case, both groups would have more long-lived lymphocytes in these organs than in the bone marrow and thymus.

Table 1. CALCULATED PERCENTAGES AND ABSOLUTE NUMBERS OF LABELED AND UNLABELED LYMPHOCYTES IN THE BLOOD

| Days after Freund's adj. injection | Percent labeled lymphocytes | | Absolute no. per c. mm. labeled lymphocytes | | Absolute no. per c. mm. unlabeled lymphocytes | |
|---|---|---|---|---|---|---|
| | control | experimental | control | experimental | control | experimental |
| 1 | 7±5.0 | 8±2.5 | 409±82 | 101±24 | 5121±1055 | 1124±276 |
| 3 | 15±3.9 | 6±2.5 | 587±144 | 136±17 | 3326±820 | 1997±246 |
| 5 | 21±4.1 | 8±3.3 | 610±231 | 210±75 | 2297±867 | 2421±875 |
| 7 | 23±7.0 | 13±6.5 | 707±114 | 333±76 | 2368±381 | 2229±513 |
| 9 | 27±4.7 | 21±3.2 | 1045±377 | 446±159 | 2825±988 | 1679±598 |
| 11 | 25±5.2 | 22±5.1 | 1075±451 | 493±192 | 3325±1354 | 1747±680 |
| 16 | 18±3.9 | 13±1.2 | 509±117 | 320±45 | 2320±572 | 2142±304 |

±standard deviation

Table 2.   BONE MARROW DIFFERENTIAL

Percentage in Smear (mean)

| Cell Classification | 11 days post adjuvant | | 16 days post adjuvant | |
|---|---|---|---|---|
| | control (4 mice) | experimental *(3 mice) | control (4 mice) | experimental (4 mice) |
| Rubriblasts | 12.3±1.7 | 11.2±2.1 | 10.5±0.1 | 3.6±0.8 |
| Rubricytes | 28.0±6.7 | 15.1±4.3 | 26.6±3.2 | 14.0±3.2 |
| Total erythropoietic cells | 40.3 | 26.3 | 37.1 | 17.6 |
| Neutrophil myelocytes and promyelocytes | 6.8±2.3 | 8.7±2.1 | 6.0±1.3 | 10.6±1.9 |
| Neutrophil segmented and metamyelocytes | 38.2±5.4 | 56.1±6.5 | 44.4±6.0 | 68.0±5.3 |
| Eosinophils (all types) | 3.5±1.8 | 2.3±1.3 | 2.9±1.1 | 0.8±0.2 |
| Total granulocytes | 48.5 | 67.1 | 53.3 | 79.4 |
| Lymphocytes (all types) | 8.4±0.7 | 3.3±0.4 | 8.8±1.2 | 2.5±0.3 |
| Blasts cells | 2.6±0.3 | 3.1±1.1 | 0.6±0.5 | 0.2±0.0 |
| Miscellaneous | 0.2±0.0 | 0.2±0.1 | 0.3±0.1 | 0.3±0.1 |

*One mouse from this group died 10 days post adjuvant

±standard deviation

Table 3.   ORGAN MEAN WEIGHTS IN MILLIGRAMS

| Organ | 11 days post adjuvant | | 16 days post adjuvant | |
|---|---|---|---|---|
| | control (4 mice) | experimental (3 mice) | control (4 mice) | experimental (4 mice) |
| thymus | 45.1±10.6 | 2.7±2.3 | 41.4±17.4 | 10.2±7.5 |
| spleen | 103.2±2.6 | 196.6±47.1 | 97.1±21.1 | 358.8±103.0 |
| liver | 968.5±179.1 | 1106.5±209.4 | 932.1±128.2 | 1344.6±266.9 |

±standard deviation

Table 4.    PERCENTAGE LABELING OF LYMPHOCYTES IN
TISSUE IMPRINTS

| Tissue | 11 days post adjuvant[1] | | 16 days post adjuvant[2] | |
|---|---|---|---|---|
| | control (4 mice) | experimental (3 mice) | control (4 mice) | experimental (4 mice) |
| bone marrow | 100±0.0 | 100±0.0 | 0±0.0 | 0±0.0 |
| thymus | 90±4.1 | 21±5.5 | 3±1.8 | 3* |
| spleen | 82±3.6 | 100±0.6 | 20±2.9 | 2±1.1 |
| lymph nodes | 64±8.0 | 98±0.8 | 28±5.4 | 15±2.9 |

[1] 12 hours after last injection of tritiated thymidine
[2] 5½ days after last injection of tritiated thymidine

* Two of the thymus imprints in this group contained no lymphocytes
since four thymus tissue imprinted poorly, only 100 lymphocytes were
counted.

± standard deviation

## DISCUSSION

This study demonstrates a lack of labeling in the peripheral blood lymphocytes of mice which received complete Freund's adjuvant and tritiated thymidine as compared to mice which received only tritiated thymidine. This lack of labeling may be the result of short-lived lymphocytes being lost from the peripheral blood; lack of labeling at the cellular level; or the adjuvant-induced peritonitis preventing the absorbtion of the labeling material.

The findings of this study regarding two populations of lymphocytes in the tissue of BALB/c/jax mice tend to support the findings of Everett, et al. (28) in the Lewis rats, and Denman, et al. (21) in the C57 Bl mouse. The following table is a summary of these findings.

Percentage of labeled lymphocytes
found in tissues of control animals

| Tissue | BALB/c/jax mice | Lewis rats | C57 Bl mouse |
|--------|------------------|------------|--------------|
| Peripheral blood | 27 | 35 | 35 |
| Bone Marrow | 100 | 100 | 88 |
| Thymus | 90 | 90 | 100 |
| Spleen | 82 | 70 | 33 |
| Lymph nodes | 64 | 25 | 50 |

The areas of greatest disparity occur between the values given for the spleen and lymph nodes which are considered to be reactive areas. These may be accounted for by species difference and/or a different schedule of tritiated thymidine administration. While Everett, et al. (28)

injected every 6 hours, both this study and Denman, et al. (21) injected every 12 hours. It may also be noted that Everett, et al. (28) calculated a 5 day life span for short-lived lymphocytes whereas this study and Denman, et al. (21) calculated a life span of approximately 7 days.

Also substantiated in this study were Morton and Siegel's (64) observations of peripheral lymphopenia, evidence of wasting, increased neutrophils, increased spleen weights, and thymic involution followed by weight gain after a single intraperitoneal injection of complete Freund's adjuvant in BALB/c/jax mice. The dramatic drop of peripheral blood lymphocytes along with the seeming incongruity of increased antibody titers to bovine serum albumin noted by Morton and Siegel (64) prompted this further investigation to determine which, if either, of the two lymphocyte populations was affected.

A single injection of complete Freund's adjuvant given to BALB/c experimental mice caused a severe drop in the circulating lymphocyte count within 24 hours. It also altered the ratio of labeled to unlabeled cells and the slope of the labeling curve. After 12½ days of intermittent injections of tritiated thymidine, approximately 25% of the circulating lymphocytes were labeled. Those that were unlabeled must have undergone their last division more than 12½ days previously, and were older cells. Those that were labeled must have been synthesizing DNA at some time after the commencement of injections and were, therefore, younger than 12½ days old. Since the mice which

received adjuvant manifested a decrease in the percentage of labeled lymphocytes it is assumed that short-lived lymphocytes decreased in the peripheral blood.

There are at least three explanations for this phenomenon: 1) short-lived lymphocytes were more susceptible to destruction by the experimental procedure than the older cells, 2) short-lived lymphocytes were not being produced, and/or 3) short-lived lymphocytes were being preferentially lost from the peripheral blood into the tissues.

1) If radiation damage from the labeled isotope had occurred in significant amounts, both the control and experimental should have been equally affected. Had there been excess fragility in the circulating lymphocytes of the animals receiving adjuvant, it would have been difficult to ascertain. However, no smudge or basket cells were observed in either the control or experimental animals. The stress produced by the injection of adjuvant may have stimulated an increased production of adrenal corticosteroids. Thus a cortisol-induced suppression of lymphopoiesis could account for the observed lymphopenia.

It has been shown that these hormones do not destroy all lymphocytes, but rather selectively eliminate only short-lived lymphocytes (17, 31). The regions associated with rapid lymphocyte proliferation and turnover; i.e. thymus, bone marrow, and germinal centers of lymph nodes and spleen, are the regions most severely depleted by cortisone treatment (13, 17, 23, 24, 47, 63). Significantly, these regions are populated by short-lived lymphocytes (14, 16, 28, 29).

Schlesinger and Mark (80) observed a wasting syndrome, similar to that developed in runt disease and in the post-thymectomy syndrome, following a single injection of 0.25 mg of cortisol acetate in young mice. Decreased dosage or older animals at the time of injection lessened the severity of the disease. Thymic atrophy was observed within one day of cortisol injection.

Following the administration of adjuvant, the reduced number of short-lived lymphocytes and wasting syndrome accompanied by thymic atrophy may be speculated to be the result of increased amounts of natural adrenal hormones due to stress. Regarding stress, the unexplained fluctuation in the control mice lymphocyte count may have been caused by a slight increase in their hormone levels due to frequent handling.

2) Short-lived lymphocytes are produced in the thymus, bone marrow and germinal centers (14, 16, 28, 29). Initially the proliferation capacity of these organs may have been impaired and consequently the cause of the decrease in short-lived lymphocytes. But because the experimental animals followed their decrease in short-lived lymphocytes by a steep rise approaching control values the impairment was only temporary or incomplete. The histology of the tissue taken after the rise shows that the germinal centers were quite actively producing cells while the thymus and bone marrow were depleted of lymphocytes. Because no tissue was examined during the first part of the experiment when the short-lived lymphocytes were diminished from the peripheral blood, it can not be stated unequi-

vocally that short-lived lymphocytes were not being produced. Also, the lack of lymphocytes in the bone marrow can not be used as an argument for non-production, because good results were not obtained in the control mice to support marrow production of lymphocytes in these mice. Thymic involution may have been the cause of short-lived lymphocyte depletion in the peripheral blood.

Miller (59, 60) demonstrated that thymectomy in the immediate neonatal period was associated with a severe depletion in the lymphocyte population. Others, including Dalmasso, et al. (20) and Arnason, et al. (2) likewise observed that rats thymectomized at birth show a striking decrease in the level of circulating lymphocytes. Thymectomy in adult animals also is fallowed by a lymphopenia (7, 57, 65, 91, 92) although the decrease in lymphocytes is not as drastic as it is in neonatally thymectomized animals, and occurs after a prolonged interval.

Using radioautographic techniques and multiple injections of tritiated thymidine to study the lymphocyte turnover in neonatally thymectomized animals, Rieke and Schwarz (77) concluded that the difference in the total number of lymphocytes between experimental and control animals was almost entirely due to a reduction in the number of long-lived lymphocytes. They further concluded that, since both tissue and circulating short-lived lymphocytes appeared to be present in near normal numbers, it seems likely that these cells were derived from lymphopoietic centers other than the thymus.

Procedures which deplete animals of long-lived recirculating lymphocytes or cause a drastic reduction in number, such as prolonged thoracic duct drainage, neonatal thymectomy, or irradiation, also render the animals immunologically incompetent (31). Gowans, et al. (39) and McGregor and Gowans (56) demonstrated that chronic drainage of lymph and cells from a thoracic duct fistula in rats severely depressed or abolished the primary immune response to sheep erythrocytes. In Morton and Siegel's (64) experiment using Freund's adjuvant to induce wasting in mice, an absolute lymphopenia was associated with enhanced production of antibody to bovine serum albumin.

Since thymectomy is associated with decreased numbers of long-lived lymphocytes and depressed antibody production, while adjuvant-induced thymic atrophy is associated with decreased numbers of short-lived lymphocytes and enhanced antibody production, the two situations are not comparable. Consequently, thymic involution probably is not the cause of the decrease in short-lived lymphocytes in these animals.

Adjuvant treated mice resemble neonatally thymectomized mice in manifestations of wasting disease and anemia. Wasting syndromes occur in the following:

a. new born mice injected with allogenic lymphoid cells (8, 36, 48, 83);

b. $F_1$ hybrids injected with parental lymphoid cells (52, 87);

c. lethally irradiated mice injected with allogenic bone marrow cells (3, 86, 90);

    d.   mice thymectomized shortly after birth (60, 70, 72);

    e.   mice inoculated at birth with polyoma virus (53);

    f.   mice injected with cortisol acetate (80);

    g.   mice injected with sterile bacterial vaccines (25);

    h.   mice injected with complete Freund's adjuvant (64).

Features these experimental animals have in common are ruffled fur, hunched posture, diarrhea, weight loss and atrophy of the thymus. Whether the underlying mechanism in all cases is the same remains to be determined. There is no evidence of any acute infectious process, but the possibility of a low-grade chronic infectious process has not been eliminated. The premise that lymphocytes may exert a nutritional or "trophic" function has been recently revived (15, 32, 55). Accordingly, lymphocytes may provide large fragments of nucleic acids or nucleoproteins to be used during the formation of new cells (44, 46), and/or provide building substances for synthetic processes (76). Withdrawal of this influence, according to Gowans (40) and Loutit (55), may lead to wasting diseases. The nature of the hemolytic anemia in the experimental group is unknown. In as much as hemolytic anemia has been shown to accompany the wasting disease resulting from neonatal thymectomy (22, 94), a common mechanism may be operative.

    3)  The third contention, that short-lived lymphocytes were being preferentially lost from the peripheral blood into the tissues, is equally difficult to substantiate. Since there was active lymphopoiesis in the lymph nodes and spleen, it cannot be stated with certainty that the increased labeling in the tissue imprints of these

organs was due to migration of short-lived lymphocytes from the peripheral blood. However, the short-lived lymphocyte population may have been drawn to the site of the adjuvant injections.

Many suggestions have been made relating to the mode of activity of Freund's complete adjuvant. The injection of the oil-water-lanolin-tubercle bacilli mixture of Freund produces a sterile abscess which is irritating, and from which the antigen is released slowly (50). The primary factor would appear to be the characteristic granulomatous response to the myobacteria at the inoculating site, and especially the proliferation of large mononuclear cells in intimate contact with antigen (50). It is probable that the short-lived lymphocytes are drawn from both the peripheral blood and thymus to the peritoneal cavity to aid in the action of the adjuvant. In addition to the response at the inoculating site, droplets of emulsion are disseminated from the inoculation sites to the regional lymph nodes with the formation of multiple granulomata (12). There is also systemic spread of these droplets with the establishment of multiple foci in spleen and lymph nodes (12). The participation of short-lived lymphocytes in these adjuvant areas would be in keeping with Craddock's (17) theory that short-lived lymphocytes are involved in the earliest phases of particle trapping, phagocytosis, and antigen processing.

The emphasis of this discussion heretofore has been on the assumption that the lack of labeling is due to a loss of short-lived lymphocytes from the peripheral blood. However the two remaining alternatives should be discussed.

In regard to lack of labeling at the cellular level, the assumption has been made that each newborn cell is labeled. If this is not the case, even though tritiated thymidine is available, perhaps some lymphocytes do not use it in DNA synthesis, or perhaps it does not reach areas of lymphocyte production. However, at present, there is no evidence to support either of these alternative assumptions.

In order to correct the shortcomings of the experimental design in relation to the effect of the peritonitis on the absorption of the labeling material, the following suggestions are offered: inject the tritiated thymidine by a different route, such as intravenously; tally the percent of labeled neutrophils, which would label rapidly and remain labeled (78) if the tritiated thymidine is available; produce a peritonitis by some other means and check for labeling of lymphocytes in the peripheral blood; and/or investigate the effect of decreasing doses of complete Freund's adjuvant in order to achieve a less sever peritonitis without sacrificing the lymphopenia.

## SUMMARY

Seven week old BALB/c/jax mice receiving a single intraperitoneal injection of complete Freund's adjuvant manifested a decrease in the labeling of lymphocytes in the peripheral blood. Autoradiographic technique and injections of tritiated thymidine at 12 hour intervals for $12\frac{1}{2}$ days were employed. In addition to the lack of labeled lymphocytes, lymphopenia, wasting disease, anemia, granulocytosis, thymic atrophy, and increased spleen and liver weights were observed. Of the several explainations for the lack of labeling of lymphocytes discussed, the hypothesis implying that short-lived lymphocytes were being lost from the peripheral blood was favored. Inconclusive evidence points toward either a cortisol-induced stress factor or migration of lymphocytes to areas of adjuvant induced activity as being responsible for the decrease in the short-lived lymphocytes.

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## PLATE I

Labeled leukocytes from peripheral blood smear of complete Freund's adjuvant treated BALB/c/jax mouse which had received tritiated thymidine every 12 hours for $12\frac{1}{2}$ days. Reading from top to bottom, the first, second and fourth are lymphocytes, the third is a neutrophil.

PLATE II
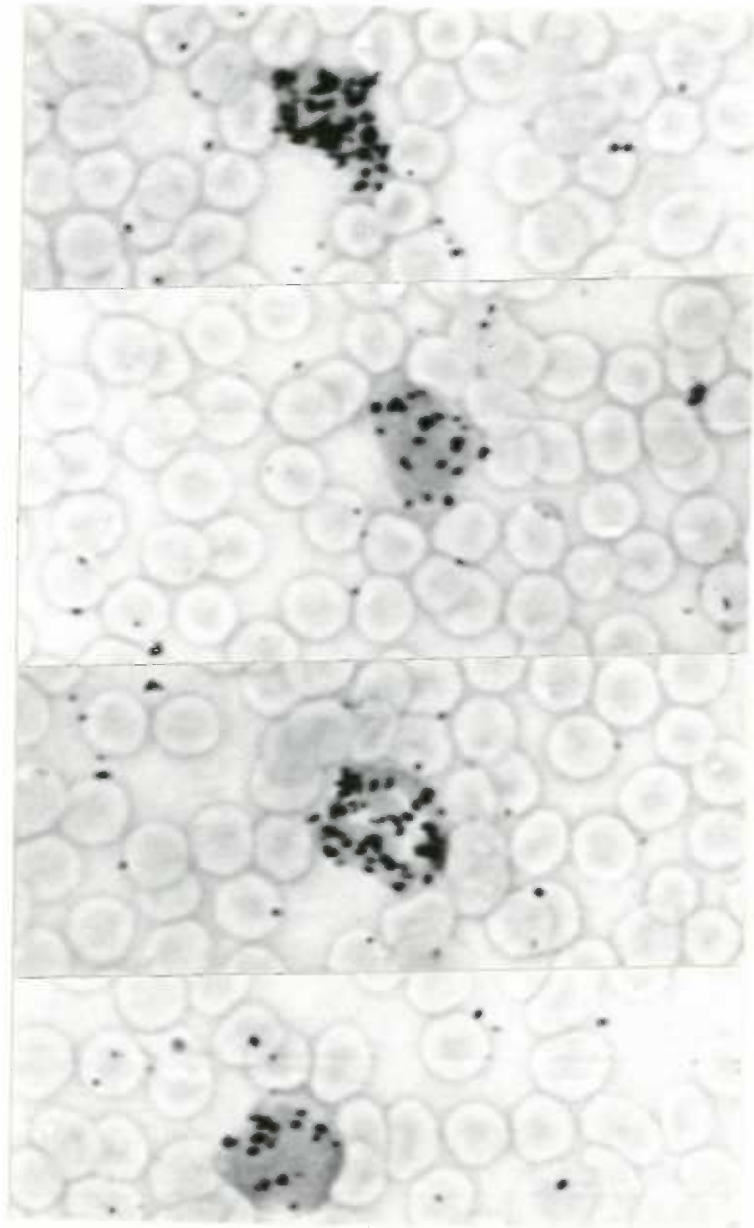
Fig. 1.   Normal BALB/c/jax mouse.   Seven weeks old and weigh-

         ing 17 gm.

Fig. 2.   BALB/c/jax mouse 24 hours after having received

         complete Freund's adjuvant.   The mouse weighed 18

         grams before receiving adjuvant.   At the time of

         the picture the mouse weighed 15.5 gm.   Note ruffled

         fur and hunched posture.
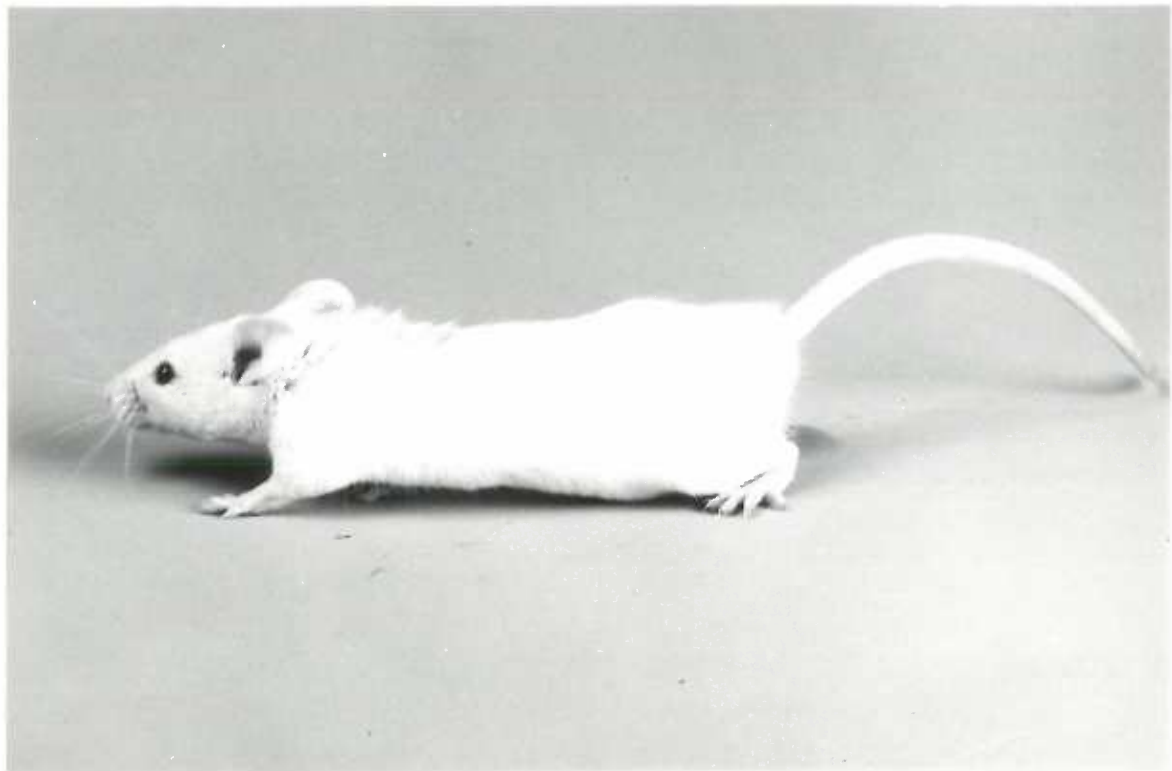
PLATE III

A comparison of the weights, volume of packed red cells, neutrophils and lympho-

cytes from the control BALB/c/jax mice (∆) and adjuvant treated BALB/c/jax mice (•).

Weight differences between the control and experimental mice were noted at 24

hours post-adjuvant. At which time the experimental group manifested an average

weight-loss of 3 grams, and continued to decline steadily until day 7 post-adjuvant.

Subsequently, their weight increased as a group, and approached control values after

day 11 post-adjuvant.

Whereas the control mice showed constant variation in volume of packed red cells,

the experimental mice declined until day 11 post-adjuvant, at which time their mean

was 10 points below the control mean.

The neutrophils of the control mice remained constant, while those of the experi-

mental mice climbed to a high of 13,500/cu. mm.

The absolute lymphocyte count of the control animals showed an inexplainable drop

after the 1st day post-adjuvant, while the experimental group showed a severe lympho-

penia and remained lower than the control group throughout the experiment.
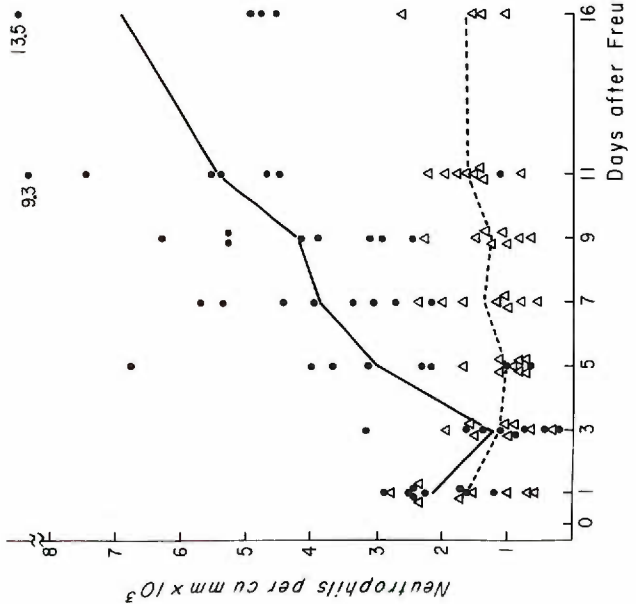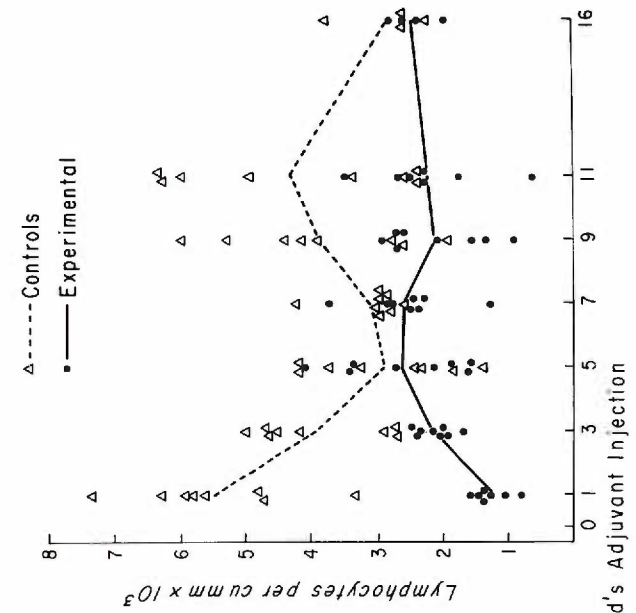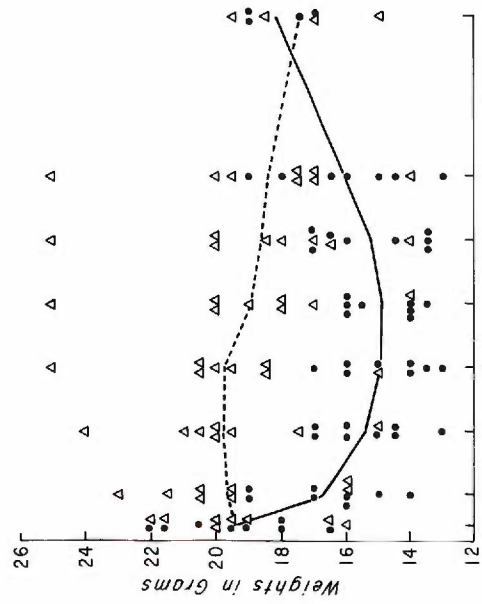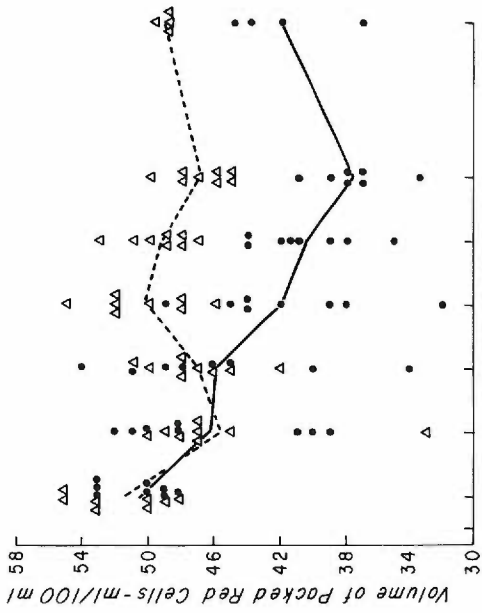
## PLATE IV

Rate of appearance of labeled lymphocytes in the blood of BALB/c/jax mice. All mice received tritiated thymidine (0.5 μc per gram body weight) by intraperitoneal injection at 12 hour intervals for $12\frac{1}{2}$ days.

The percentage of labeled lymphocytes in the control group rose steadily between the first and fifth days; but there was no rise in labeling until the fifth day in the mice which received complete Freund's adjuvant. The lack of labeling in the experimental group was taken as evidence of loss of short-lived lymphocytes from the peripheral blood.
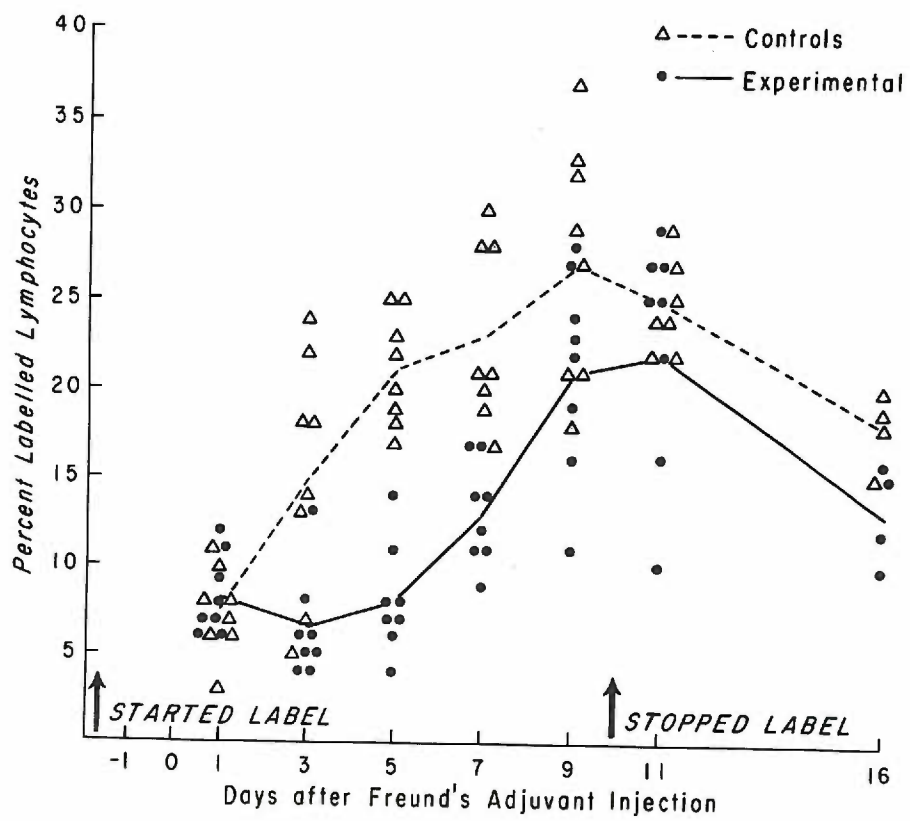
PLATE V

A portion of thymus from a control BALB/c/jax mouse.  Note the
distinct arrangement of cortex which consists of numerous dark stain-
ing lymphocytes with an occasional mitotic figure.  The medulla is
pale and shows loosely arranged reticuloendothelial cells and large
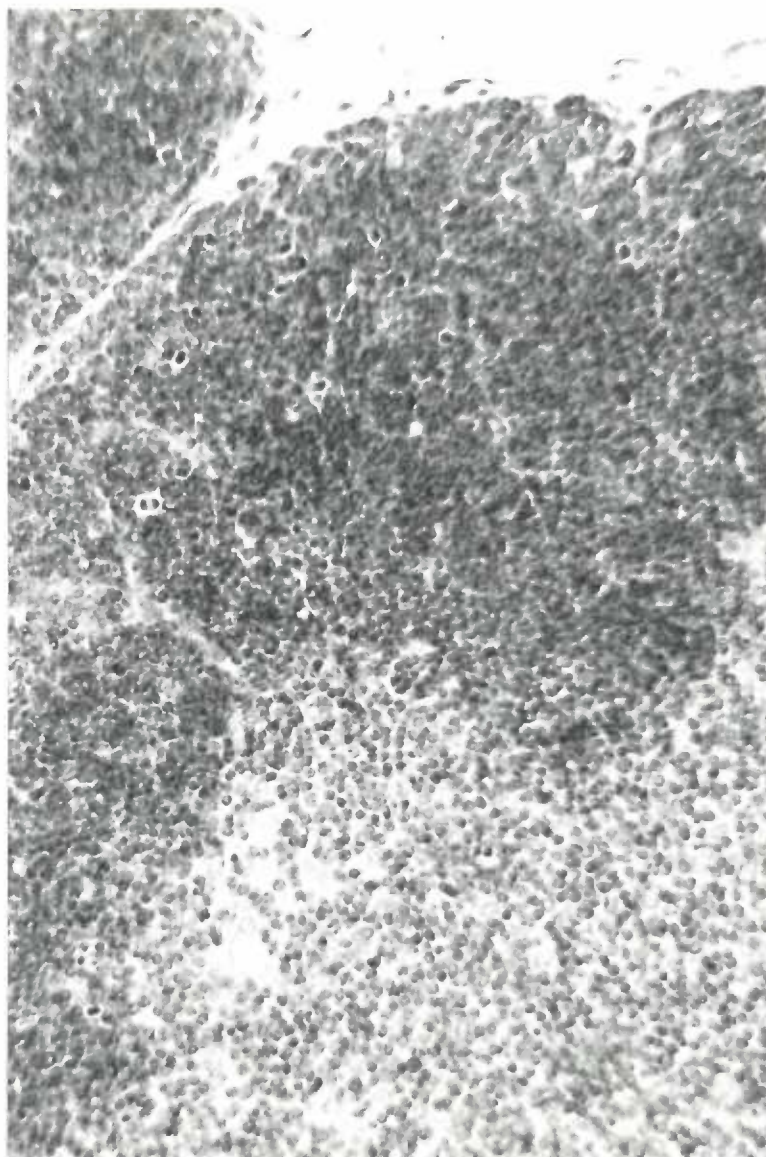lymphocytes.   (160 X)

PLATE VI

Fig. 1.   Portion of thymus from experimental BALB/c/jax mouse
sacrificed at 11 days post adjuvant.   Note the paucity
of lymphocytes from the extreme peripheral edge of
the cortex.   In the remaining cortex large multinucleat-
ed giant cells can be seen.   (200 X)

Fig. 2.   A high power magnification of the experimental thymus
tissue, showing border between lymphocyte populated
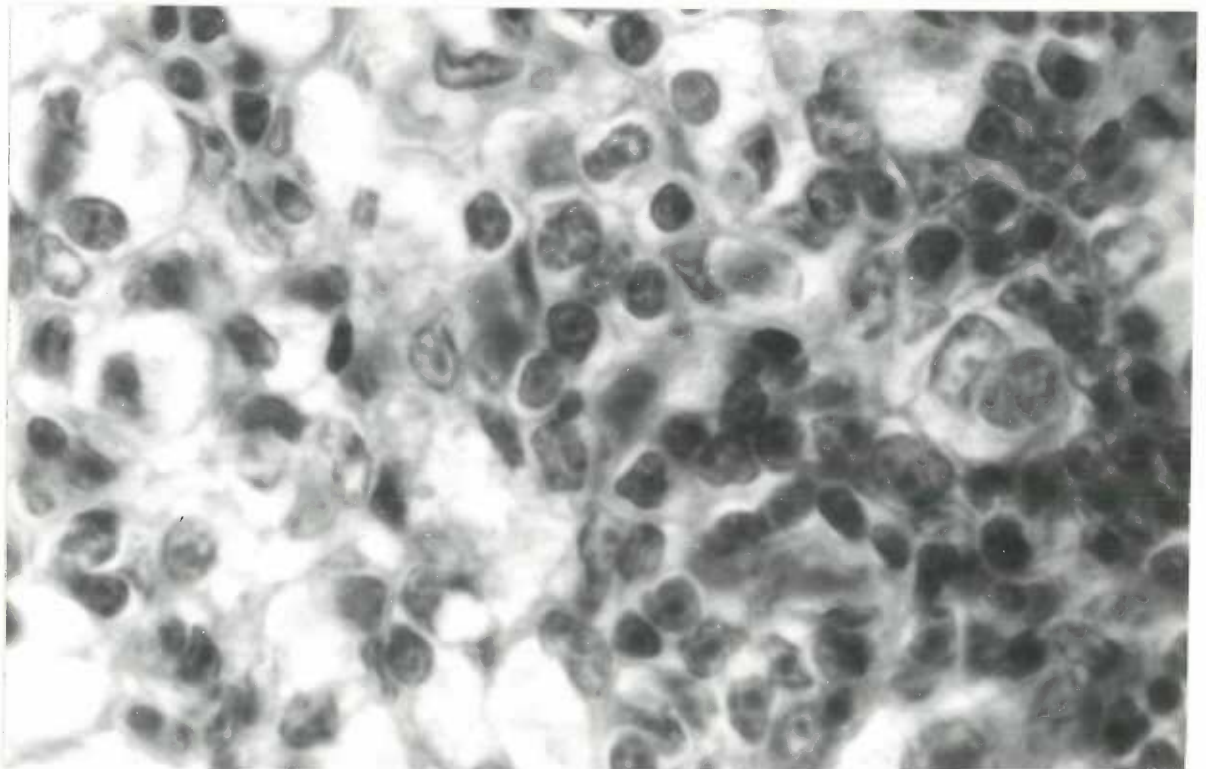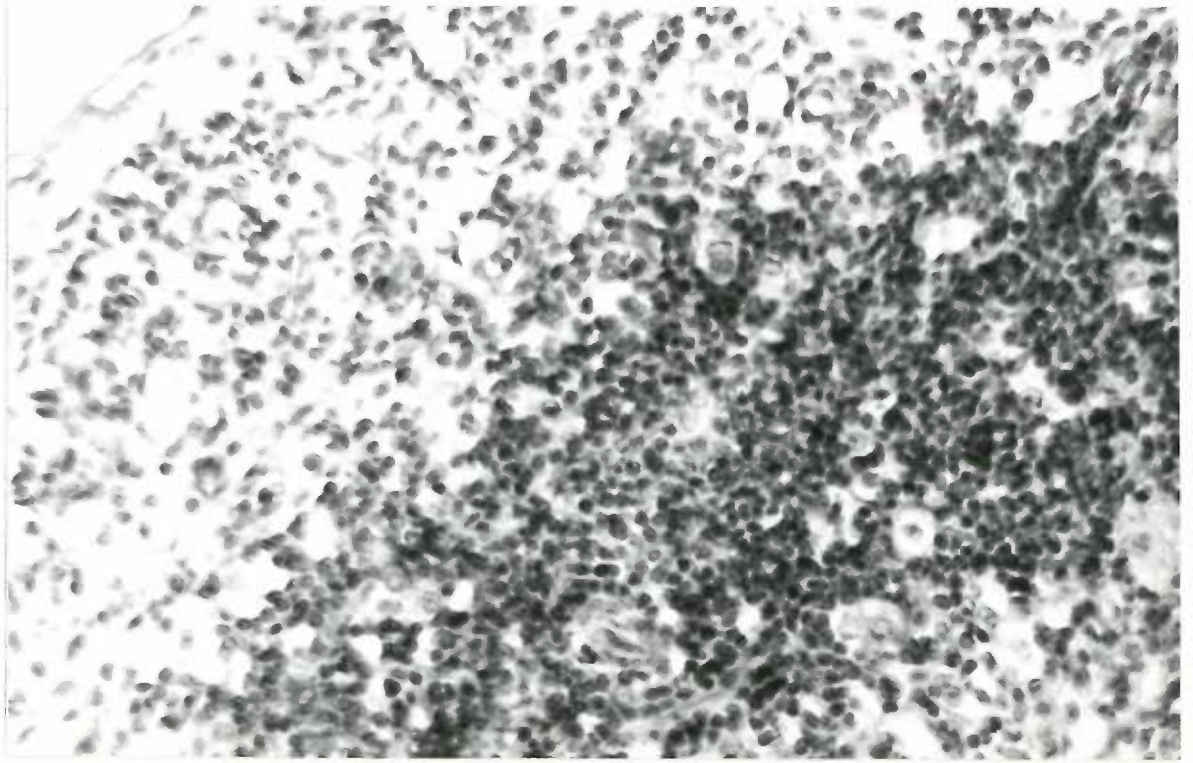cortex on right and lymphocyte depleted cortex on left.
(800 X)

PLATE VII

Fig. 1.   Spleen of BALB/c/jax mouse 11 days post complete
          Freund's adjuvant injection.   The clusters of dark
          staining cells in the red pulp represent myeloid
          and erythrocytic cells undergoing maturation.   (200 X)

Fig. 2.   A high power magnification of the above spleen, in-
          dicating red pulp activity with megakaryocytes
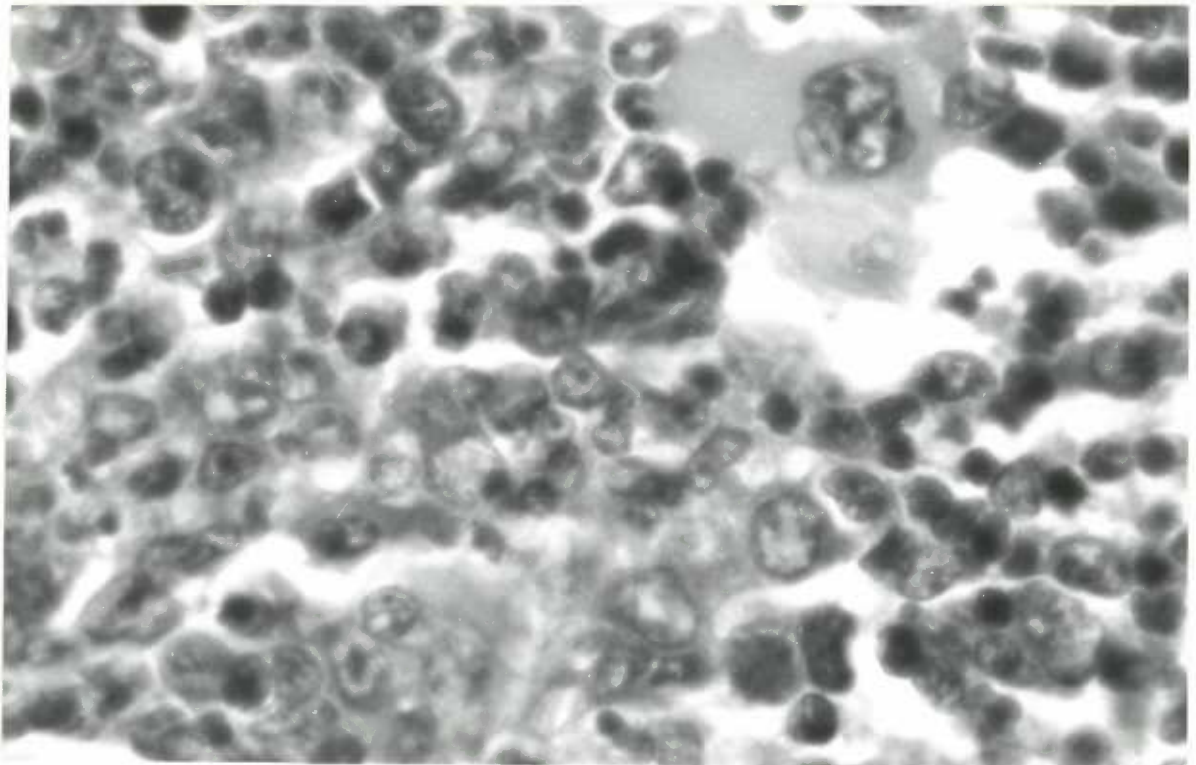          prominent.   (800 X)

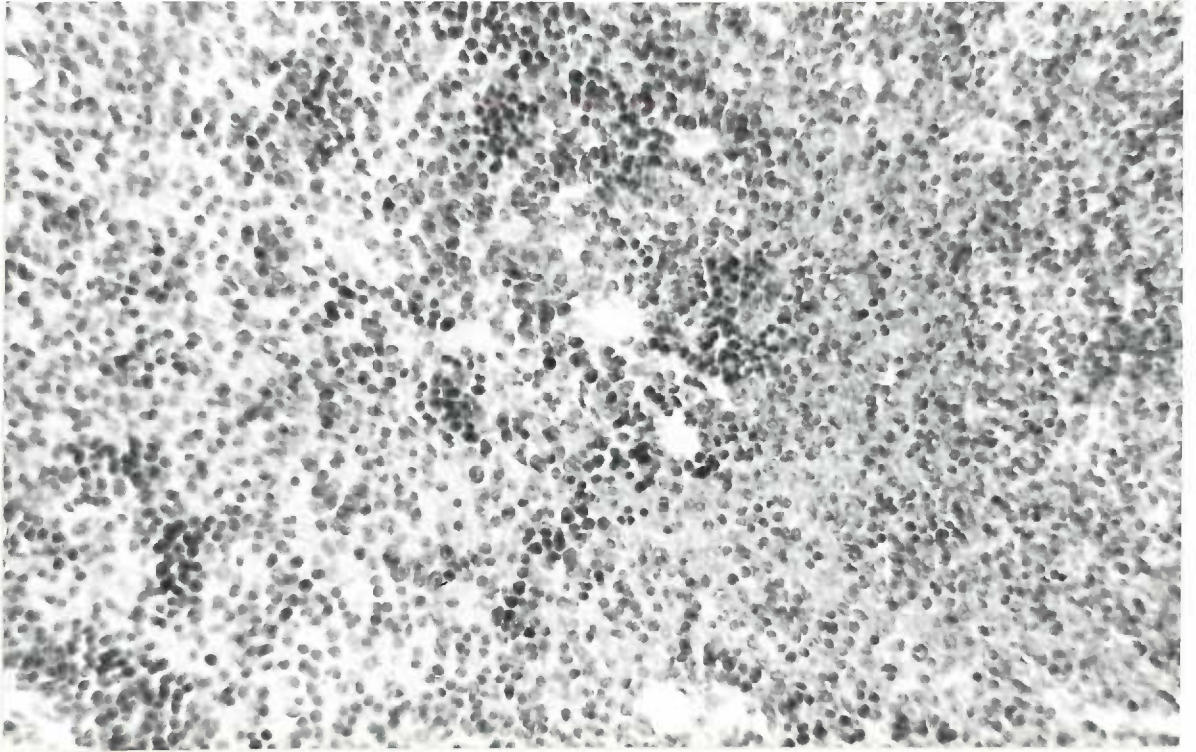PLATE VIII

Fig. 1.     Lymph node from BALB/c/jax mouse 11 days post
            complete Freund's adjuvant injection.  Formative
            granuloma is shown at the right consisting of histio-
            cytes and multinucleated giant cells surrounded by
            lymphocytes and plasma cells.  (200 X)

Fig. 2.     High power magnification of above node at rim of
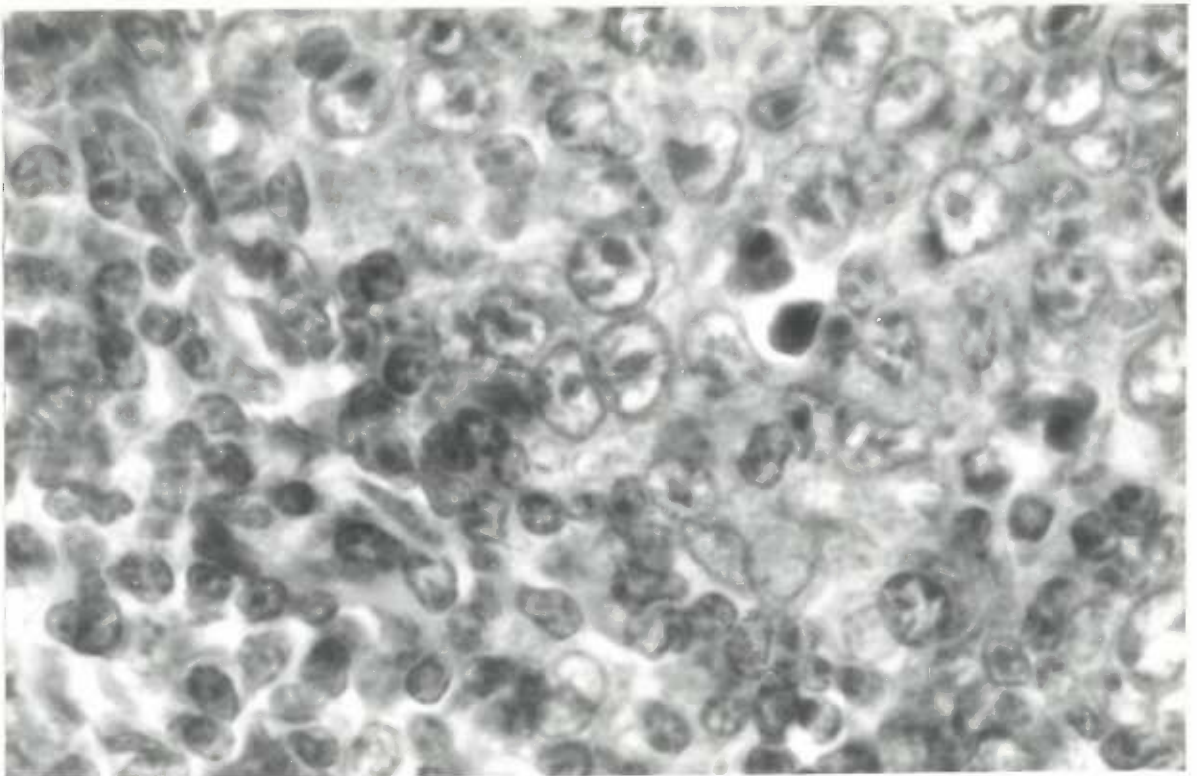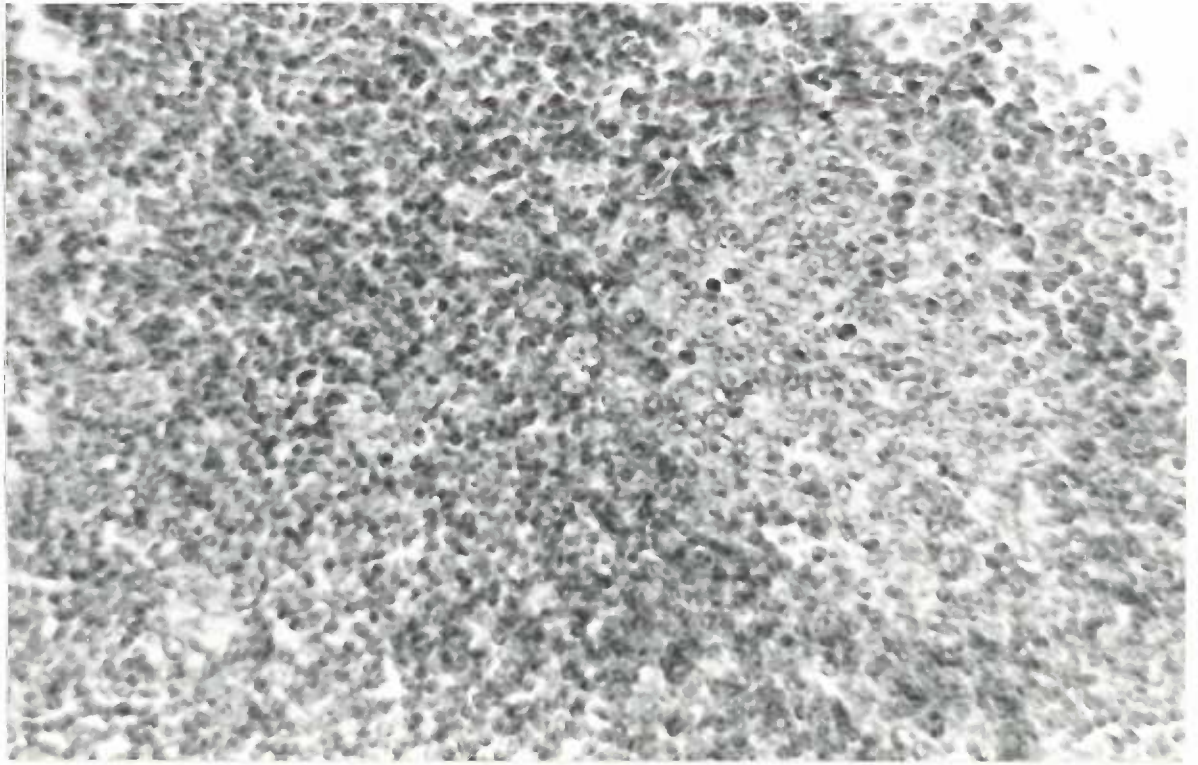            granuloma formation.  (800 X)

PLATE IX

Fig. 1.   Portion of BALB/c/jax mouse liver 11 days post
          complete Freund's adjuvant injection, showing tre-
          mendous inflammatory cell infiltrate.   (200 X)

Fig. 2.   High power magnification from above section of liver.
          (800 X)