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A STUDY OF HEMOPOIETIC TISSUE AND SKIN ALLOGRAFT REJECTION IN THE NEWT, NOTOPHTHALMUS VIRIDESCENS.

The Ohio State University, Ph.D., 1970
Anatomy

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A STUDY OF HEMOPOIETIC TISSUE AND SKIN ALLOGRAFT REJECTION

IN THE NEWT, NOTOPHALMUS VIRIDESCENS

DISSertation

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

By


* * * * *

The Ohio State University
1970

Approved by

Ronald J. Giffords
Adviser
Department of Anatomy
ACKNOWLEDGMENTS

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PART I

HEMOPOIETIC TISSUE IN THE ADULT NEWT, NOTOPHTHALMUS VIRIDESCENS
INTRODUCTION

The amphibian lymphomyeloid system includes the thymus, spleen, bone marrow and lymphocytic accumulations such as the jugular body, paracoracid body, hemal nodes and the propericardial body (Baculi et al., '70; Baculi and Cooper, '68b). The most complex lymphomyeloid systems of the amphibian class are found in anurans since many anuran species possess extra-thymic lymphocytic accumulations and the bone marrow of most anurans is active at some time during their life cycles (Jordan, '38).

In contrast, the urodele lymphomyeloid system is not as complex as that observed in anurans because; (1) no extra-thymic accumulations of lymphocytes have been described in the species studied thus far; and (2) with the exception of Plethodon cinereus (Jordan, '38), no hemopoietic activity has been seen in the bone marrow at any stage in development.

The most comprehensive morphological investigations of the hemopoietic tissues of urodeles have utilized Ambystoma punctatum (Baldwin, '18), Ambystoma mexicanum (Klug, '67) and the newt, Notophthalmus viridescens (Jordan and Speidel, '24, '30; Tooze and Davies, '67, '68) as models. Previous studies of the hemopoietic activity of the newt, Notophthalmus viridescens, have revealed that
the spleen was lymphopoietic, thrombopoietic and erythropoietic
(Jordan and Speidel, '24, '30; Tooze and Davies, '67, '68), whereas
the capsule of the liver was entirely granulopoietic (Jordan and
Speidel, '24, '30). In addition, no hemopoietic activity was
observed in the bone marrow of the newt (Jordan and Speidel, '24,
'30; Tooze and Davies, '67, '68) and very little lymphopoiesis
was seen in the lamina propria of the intestine (Jordan and Speidel,
'24).

A detailed morphological study of the thymus, the only other
hemopoietic organ of the adult newt, has, to our knowledge, never
been presented. The primary objective of this study was to complete
the histological picture of the hemopoietic organs of the newt by
describing the histology of the thymus and to compare the cells
observed in the thymus with the same types of cells in the spleen
and liver. A complete histological picture was, in turn, necessary
in order to provide a baseline for subsequent studies on the in vivo
effects of skin allografting and the administration of Phytohemagglutinin
on the hemopoietic tissues.
MATERIALS AND METHODS

Newts obtained from Glenn Gentry (Donelson, Tennessee) were maintained in an aquarium at a constant temperature of 23°C. until studies were begun. The newts were fed small pieces of liver two to three times a week before the studies were initiated and adlibitum during the studies.

Male and female normal adult newts were fixed in formol Zenker's solution. The newts were cut into small pieces from anterior to posterior and double embedded. Five micra serial sections were cut and stained with Harris' hematoxylin and eosin and Periodic Acid Schiff's (PAS).

In addition, the thymuses, spleens, livers, forelimbs, hindlimbs, and small intestines were removed from male and female normal adult newts, fixed in formol-sublimate-acetic acid (FSA) and embedded in plastic using a modification of the technique of Ruddell ('67) [Appendix A]. Two micra sections were cut and some stained with a modification of Harris' hematoxylin and eosin (Appendix B) and others with a 0.3% aqueous solution of toluidine blue.

The thymuses were removed from the newts as previously described (Rubens and Balls, '67) by carefully picking up the skin slightly dorsal and 5-10 mm posterior to the angle of the jaw (Fig. 1) with jewelers forceps and cutting the epidermis and underlying connective tissue around the forceps with iridectomy scissors so as to remove a piece of tissue 2-3 mm in diameter containing the thymus.
RESULTS

Thymus

The dorsally positioned thymus consisted of three lobes (Fig. 2) each of which appeared to be at least partially encapsulated judging by the orientation of fibroblasts (Fig. 3) and collagen fibers (Fig. 4). However, the absence of PAS reactivity in the area adjacent to the peripheral parenchyma of the thymus suggested that the capsule of the thymus was not extensive.

The parenchyma of the thymus of the newt exhibited no clear demarcation of cortex and medulla (Figs. 2, 5). Stromal cells characterized by; (1) a moderate amount of eosinophilic cytoplasm (Fig. 6); (2) a centrally located nucleus with a fine chromatin pattern (Figs. 6, 7); (3) two to five nucleoli (Figs. 6, 7); and (4) a sparse amount of nuclear membrane-associated chromatin (Figs. 6, 7) as well as small lymphocytes (Figs. 8, 9) which were spherical (5μ x 5μ) or ovoid (5μ x 8μ)-shaped cells possessing a more dense chromatin pattern with more apparent nuclear membrane-associated chromatin and fewer nucleoli than were observed in stromal cells were both distributed throughout the parenchyma of the thymus (Fig. 10).

A number of cells with ovoid-shaped nuclei (7μ x 9μ) [Fig. 11] and elongated-shaped nuclei (7μ x 14μ) [Fig. 12] were observed throughout the parenchyma of the thymus (Fig. 13). The chromatin of
these cells was generally organized into elongated strands in much
the same pattern as was observed in the mature erythrocytes (Fig. 14)
which were seen in the parenchyma of the thymus. However, since the
nuclei were rarely larger than the nuclei of mature erythrocytes, it
was judged unlikely that the cells were precursors of mature
erthrocytes. These cells were observed in every thymus examined,
but generally their central distribution was not as obvious as is
indicated by figure 13.

Melanocytes, irregularly-shaped cells (25μ-30μ) containing
many small granules (0.5μ in diameter) in the cytoplasm and the
cytoplasmic processes (Fig. 15), were observed immediately peripheral
to the capsule of the thymus with several usually seen in each section
(Fig. 5). Elongated strands of clumped chromatin were seen next to
the nuclear membrane of melanocytes (Fig. 15). The nucleo-cytoplasmic
ratio of most of these cells was approximately one to six.

A few mature granulocytes were observed adjacent to the
parenchyma of the thymus (Figs. 4, 16), but none were observed in the
thymus per se.

The capillaries which were in the parenchyma of the thymus
were composed of a single layer of endothelial cells. There were no
Hassall's corpuscles, tissue mast cells, myoid cells or large
lymphocytes observed in the parenchyma of the thymus.

Spleen

The spleen of the newt was bordered by the left lobe of the
liver, the stomach, and the left lung (Fig. 17) and was partially
supported in the peritoneal cavity by the dorsal mesogastric ligament (Fig. 17). The spleen was encapsulated by a serosal membrane consisting of a few collagen fibers and a layer of mesothelial cells (Fig. 18) and was composed of a cortex consisting of red pulp and a medulla of white pulp (Fig. 19). There were no lymphocytic follicles in the white pulp (Fig. 19).

Developing erythrocytes (Fig. 19) and tissue mast cells (Fig. 20) were most prevalent in the red pulp (cortex) of the spleen. Large and small lymphocytes (Fig. 21) were observed in the medulla of the spleen. The large lymphocytes (18μ-22μ) were characterized by a moderate amount of basophilic cytoplasm in contrast to small lymphocytes (8μ-10μ) which possessed very little cytoplasm. In addition, the chromatin pattern was generally more dense and clumping of chromatin more prevalent in small lymphocytes than in large lymphocytes.

The folded appearance of the nuclear membrane as demonstrated by the membrane-associated chromatin characterized thrombocytes which were most frequently observed in the medulla of the spleen (Fig. 22).

Free reticulo-endothelial cells, approximately 20μ in diameter were encountered occasionally in the cortex of the spleen. These cells were distinguished by the presence of ingested material in the cytoplasm (Fig. 23).

Liver, intestine and bone marrow

Aside from developing neutrophils (Fig. 24) and eosinophils, cells resembling small lymphocytes were occasionally seen in the capsule
of the liver (Fig. 25). In addition, small accumulations of large and small lymphocytes were observed in the lamina propria of the intestine (Figs. 26, 27). There was no hemopoietic activity in the bone marrow of the normal adult newt (Fig. 28); the marrow cavity was filled entirely with fat cells and fibroblasts.
The observations presented in this paper indicate that lymphopoiesis in the adult newt is most likely limited to the thymus, the medulla of the spleen and the lamina propria of the intestine. This limited distribution of lymphocytic tissue distinguishes the newt from anuran amphibians such as the bullfrog (Cooper, '67; Baculi and Cooper, '67) the midwife toad (Du Pasquier, '68), the South African clawed toad (Manning and Horton, '69; Baculi and Cooper, '68a), the fire frog, the leopard frog, the burrowing frog, the marine toad and the tree frog (Baculi et al., '70) [all of which possess lymphocytic accumulations in areas other than the thymus, the spleen and the lamina propria of the intestine] as well as reptiles (Good et al., '66) and mammals (Weiss, '66). The observations of the other hemopoietic tissue presented here do not differ markedly from those presented by others (Jordan and Speidel, '24, '30; Tooze and Davies, '67, '68).

The dorsal location of the thymus and its three-lobed composition is consistent with the assertion that it arises embryonically as a dorsal evagination of the third, fourth and fifth pharyngeal pouches (Noble, '31). The absence of cortical and medullary regions indicated by the equal distribution of cells throughout the parenchyma of the thymus is in agreement with the
observations of Klug ('67) on the thymus of *Ambystoma mexicanum*. The absence of an observable cortex and medulla differentiates the thymus of urodele amphibians from that of anuran amphibians (Baculi et al., '70).

The fact that mature erythrocytes, granulocytes, melanocytes, stromal cells and small lymphocytes are found within the parenchyma of the thymus, or closely associated with it, distinguishes the thymus of urodele amphibians from the thymus of anuran amphibians as well as the other vertebrate classes. The cells of the thymus of the newt with ovoid and spherical-shaped nuclei were probably small lymphocytes whose unusual shapes indicated that they were undergoing ameboid movement at the time of fixation. An insufficient number of granulocytes were observed in order to determine if they were being produced in the thymus as has been suggested by Noble ('31), or if they had arrived in the thymus from some extra-thymic source.

The capillaries observed in the thymus in the present study, composed of a single layer of endothelial cells, are similar to those in the thymus of *Ambystoma mexicanum* (Klug, '67) and were not enveloped by the epithelial-reticular cells which are characteristically associated with capillaries of the mammalian thymus (Weiss, '66).

The absence of Hassall's corpuscles noted here is consistent with the observations of Klug ('67) and together with the absence of myoid cells and a cortex and medulla tends to differentiate the thymus of the urodele amphibian from the anuran amphibian thymus (Baculi et al., '70; Törö et al., '67) and the thymus of higher vertebrates. In
contrast to the findings of Klug ('67), no tissue mast cells were found in the thymus of the newt.

Since lymphopoiesis in the adult newt is most likely limited to the thymus, the medulla of the spleen and the lamina propria of the intestine and since the newt is immunologically competent (Cohen, '66a, '66b) the thymus, the spleen and the intestine are most likely the only sources of immunocompetent cells. Since the site of production of immunocompetent cells can be delineated far more accurately than is possible in amurans, the newt provides an excellent model for further studies on the cellular interactions which occur during cellular and humoral immune responses in amphibians.
SUMMARY

Serial sections of normal adult newts (Notopthalmus viridescens) and sections of the thymuses, spleens, bone marrows, livers and intestines of newts revealed that: (1) lymphopoiesis occurred in the thymus and the medulla of the spleen; (2) erythropoiesis was taking place in the cortex of the spleen and possibly in the thymus; (3) thrombopoiesis was limited to the medulla of the spleen; (4) granulopoiesis was occurring in the capsule of the liver; (5) lymphopoietic foci were occasionally present in the lamina propria of the intestine; and (6) no hemopoietic activity occurred in the bone marrow.

Stromal cells, small lymphocytes, granulocytes, mature erythrocytes and melanocytes were observed either within the parenchyma of the thymus or closely associated with it. The urodele thymus differed from the thymus of anurans and higher vertebrates in that it lacked a cortex and a medulla, myoid cells and Hassall's corpuscles.
PLATE I
EXPLANATION OF FIGURES

1. Normal adult male newt. The thymus (T) is located slightly
dorsal and 5-10 mm posterior to the angle of the jaw.

2. Thymus showing three lobes (arrows) positioned
immediately dorsal to the epaxial musculature of an adult
newt. X 76

3. Fibroblast (F) in the capsule between two lobes of the thymus
of the newt. X 1700

4. Collagen fibers (CF) encapsulating a lobe of the thymus (T)
of the newt. A neutrophilic granulocyte (NG) is located
peripheral to the parenchyma of the thymus (T). X 1700

5. Melanocytes (M) immediately adjacent to the parenchyma of the
thymus (T). There is no clear demarcation of a cortex and a
medulla in the thymus. X 240

6. Cytoplasm (C) of a stromal cell in the thymus of the newt.
X 1700

7. Stromal cells (SC) in the parenchyma of the thymus. X 1700

8. Small lymphocytes (SL) in the thymus of the newt. X 480
PLATE II
EXPLANATION OF FIGURES

9. Small lymphocyte (arrow) in the thymus of the newt.  X 1700

10. Stromal cells (SC) and small lymphocytes (SL) are distributed throughout the parenchyma of the thymus.  X 480

11. Cells with ovoid-shaped nuclei (arrows) in the thymus of the newt.  X 1700

12. Cells with elongated-shaped nuclei (arrow) in the thymus of the newt.  X 1700

13. Central area of the thymus of the newt (arrow) composed of a large number of cells which are characterized by ovoid and elongated-shaped nuclei with a large amount of clumped chromatin.  X 480

14. Mature erythrocyte (E) in the thymus of the newt.  X 1700

15. Melanocyte (M) with a large number of small granules in the cytoplasm and the cytoplasmic processes. Strands of chromatin (arrow) adjacent to the nuclear membrane.  X 1700

16. Eosinophilic granulocyte (EG) in the outer region of the parenchyma of the thymus.  X 1700
PLATE III

EXPLANATION OF FIGURES

17. Spleen (SP) partially supported in the peritoneal cavity by the dorsal mesogastric ligament (DML). The liver (LI), lung (LU) and stomach (ST) are shown also. X 76

18. Mesothelial cell (MC) and collagen (CO) of the capsule of the spleen. X 1700

19. Cortex (SPC) and medulla (SPM) of the spleen. X 240

20. Tissue mast cell (TMC) in the cortex of the spleen. X 1700

21. Large lymphocyte (LL) and small lymphocyte (SL) in the medulla of the spleen. X 1700

22. Thrombocyte (TC) with characteristic folded appearance of the nuclear membrane-associated chromatin in the medulla of the spleen. X 1700

23. Free reticulo-endothelial cell (FRE) in the cortex of the spleen. X 1700

24. Granulocytes in various stages of development in the capsule (C) of the liver of the newt. X 240
PLATE IV
EXPLANATION OF FIGURES

25. Small lymphocyte (SL) in capsule of the liver of the newt.
X 1700

26. Lymphocytes (L) in the lamina propria of the intestine.
X 480

27. Large lymphocytes (LL) and small lymphocytes (SL) in the lamina propria of the intestine. X 1700

28. Cross-section through the humerus (HU). The marrow cavity (MC) is filled entirely with fat cells and fibroblasts. X 67
PART II

SKIN ALLOGRAFT REJECTION IN THE NEWT, NOTOPTHALMUS VIRIDESCENS,

WITH REFERENCE TO THE EFFECT OF PHYTOHEMAGGLUTININ
INTRODUCTION

The rejection of skin allografts and the distribution of lymphomyeloid tissue has been studied in many amphibian forms (Pizzarello and Wolsky, '60, [newt]; De Lenney, '61, [axolotl]; Erickson, '62, [newt]; Quinn, '64, [newt]; Cooper and Hildemann, '65, [bullfrog]; Bovbjerg, '66, [leopard frog]; Cohen, '66a, '66b, [newt]; Klug, '67, [ambystoma]; Baculi and Cooper, '68a, '68b, [South African clawed toad and bullfrog]; Horton, '69, [South African clawed toad]; Manning and Horton, '69, [South African clawed toad]. However, with the exception of one study (Cooper, '68), little attempt has been made to determine the origin of cells participating in amphibian allograft rejection.

In a previous investigation from our laboratory (Hightower, '70), it was determined that in the newt, Notophthalmus viridescens, lymphocytic tissue was limited to the thymus, the medulla of the spleen and the lamina propria of the intestine. This delineation of lymphocytic tissue offers an unusual opportunity to study these organs and the cells associated within in allograft rejection.

To this end, two objectives were established; (1) a determination of the in vivo effects of Phytohemagglutinin-P (PHA) on the spleen, the thymus, the lamina propria of the intestine and for completeness, the liver and the bone marrow (the latter two organs are usually considered hemopoietic in adult amphibians); and (2) an investigation of the effects of PHA on skin allograft
rejection in the newt. Although no investigation has been reported on the in vivo effects of PHA on amphibian hemopoietic tissue and skin allograft rejection, there exists enough evidence that PHA does affect the immunocompetence of mammals to justify using PHA for our purposes (Casciani and Cortesini, '67; Markley et al., '67a, 67b, '69; St. Pierre et al., '67; Moore and Stefani, '68; St. Pierre, '68; Lozzio et al., '69; Machado et al., '69; Ono et al., '70; Stefani and Moore, '70).
MATERIALS AND METHODS

Newts obtained from Glenn Gentry (Donelson, Tennessee) were maintained in an aquarium at a constant temperature of 23°C. until studies were begun. The newts were fed small pieces of liver two to three times a week before the studies were initiated and ad libitum during the studies.

Grafting procedures for untreated control newts

Before untreated control newts were grafted, they were anesthetized in a 0.3% aqueous solution of chlorobutanol (Penick Co., New York, New York). Full thickness dorsal skin allografts 2-3 mm in diameter were removed from a male and a female newt, placed in a drop of cold Ringer’s solution where they were flattened and then transferred to graft beds located in the ventral body walls of each of 16 (8 males and 8 females) newts (Fig. 32). As a control for infection, an autograft of dorsal skin was transferred to a graft bed located in the ventral body wall of each newt (Fig. 32). One per cent Chloromycetin Cream (Parke-Davis and Co., Detroit, Michigan) was applied to the wounded areas in an effort to minimize infection. The newts were maintained in an anesthetized state on a moist piece of filter paper for 6-18 hours and in individual dishes throughout the experiment. Approximately 5% of the grafts were lost using the procedure.
described above. In addition, about five per cent of the newts never recovered from the anesthetic.

In order to photograph and study the grafts, the newts were immobilized and grafts were flattened by placing the newts in a specially designed apparatus so that the ventral surface of the newts and the grafts were pressed against a cover slip (Fig. 33). The grafts were inspected immediately before the newts were placed in the apparatus in order to determine whether or not the procedure outlined above decreased the number of patent blood vessels in and around the graft. A decrease in blood flow was observed occasionally and in this case, either the newt was placed in a slightly larger apparatus of similar design or the position of the newt in the original apparatus was altered slightly. Grafts were examined and photographed on days 1, 4, 7, 9, 10, 16, 23, 28, 34 and 40 after grafting. Two newts (one male and one female) were sacrificed on days 1, 4, 10, 16, 23, and 40 after grafting. Immediately preceding sacrifice, grafts were photographed and the newts were weighed. After sacrifice the spleens were excised, weighed and fixed in formol-sublimate-acetic acid (FSA). The grafts, thymuses, liver, left hindlimb and intestine were then removed from each newt, fixed in FSA, embedded in plastic using a modification of the technique of Ruddell ('67) [Appendix A], sectioned at 1-2μ with a rotary microtome and stained with a modification of the Harris' hematoxylin and eosin stain (Appendix B).

PHA administration and grafting procedures for PHA-treated newts

Phytohemagglutinin-P (PHA) [Difco, Detroit, Michigan],
reconstituted with 10 ml sterile cold Ringer's solution was adjusted to a concentration of 0.85 mg/0.1 cc and injected in 0.1 ml volumes intraperitoneally into six female and nine male newts on days 1, 4 and 7. These newts received no skin allografts and thus served as PHA controls. Two newts (one male and one female) from the PHA control group were sacrificed on days 8, 11, 17, 23, 30 and 41 days after the first injection of PHA. Before sacrifice, the newts were weighed and after sacrifice spleens were excised, weighed and fixed in FSA. The thymuses, spleens, livers, left hindlimbs and intestines were processed as described above.

Two injections of PHA prepared as described above were injected intraperitoneally into a second group of seven male and seven female newts on days 1 and 4. Three days after the second injection of PHA, three grafts (one male graft, one female graft and one autograft) were applied to each newt using the procedures described in the previous section. One male and one female newt served as donors of all allografts. Grafts were inspected and photographed on days 3, 7, 12, 19, 23, 29, 35, 40 and 46 after grafting. Ten of the original 14 newts survived until termination of the experiment, 53 days after the original injection of PHA. The sex of the donor and recipient newts was recorded in order to ascertain any sexually dimorphous response to skin allograft rejection.

A third group of 10 male and 10 female newts was given three intraperitoneal injections of PHA (prepared as described above) on days 1, 4 and 7. A male allograft, a female allograft and an autograft were placed on the 10 male and 10 female newts 12 hours after the last
injection of PHA. One male and one female newt served as donors for all allografts. Thirteen male newts which received three injections of 0.1 ml cold Ringer's solution served as controls.

Grafts from this third group of newts were inspected and photographed on days 3, 4, 8, 10, 16, 23, 28, 34, and 43 after grafting. One control newt and at least one female and one male newt from the group which had received three injections of PHA were sacrificed on days 1, 4, 10, 16, 23 and 43. Newts were weighed before they were sacrificed. Spleens were excised and weighed. Grafts, spleens, livers, left hindlimbs, small intestines and thymuses were processed as described above.

A fourth group of newts (29 male newts and 9 female newts) received three intraperitoneal injections of PHA (prepared as described above) 1, 4 and 7 days after the newts were grafted with a male and a female allograft and an autograft. Sixteen of the 38 newts in this group died within 28 days after grafting. Non-treated newts received 0.1 ml cold Ringer's solution at the same time that PHA was administered to the experimental group. Grafts were inspected and photographed periodically during the course of the experiment. Control newts and male and female experimental newts possessing representative grafts were sacrificed on days 8, 10, 16, 23 and 46 after grafting. The grafts, livers, small intestines, thymuses, left hindlimbs and spleens were processed as described above.

\[ S = \sqrt{\frac{x^2}{N-1}} \]

Standard deviations were calculated by using the formula above. The two-tailed student "t" test was utilized in order to
determine the significant differences between uncorrelated means and the "f" test was used to ascertain the significant differences between standard deviations. In both the "t" test and the "f" test, significance was arbitrarily defined as "P" being less than 0.05.

A determination of the time of appearance of four stages of allograft rejection (i.e., primary vasodilation, recirculation, signs of rejection and complete rejection) was made by an examination of the surface morphology of the grafts of each group of newts. Since it was often difficult to determine precisely the time and the sequence of appearance of secondary vasodilation, stasis and hemorrhage, the onset of any one of the three phenomena was noted and recorded as "signs of rejection". After an examination of approximately 50 allografts in various stages of rejection, complete rejection was judged to occur when there was total cessation of blood flow through the graft. This definition of complete allograft rejection was utilized subsequently when complete rejection times were determined.
RESULTS

Histology of the bone marrow, intestine, liver and thymus

The bone marrow of the left tibias and femurs of control newts and newts which received PHA was found to consist almost entirely of fat cells; no hemopoiesis was observed (Fig. 34). Likewise, the histology of the lamina propria of the intestine, the capsule of the liver and the thymus was very similar in experimental newts and control newts (described in Part I).

Histology of the spleen

Forty-one spleens excised from newts at various times after they received injections of PHA (i.e., spleens excised from all newts receiving PHA whether or not the newts were grafted) were compared histologically, by weight and by sex with 19 spleens excised from control newts (i.e., newts which received either injections of 0.1 ml cold Ringers solution or no injections at all) [Appendix C].

Analysis of the spleen data revealed three significant points. First, there was no significant difference between the mean (±S.D.) weight of control spleens excised at five different time periods and all 19 control spleens. However, comparison of mean spleen weights and standard deviations of experimental spleens at five different time periods with mean spleen weight and standard deviation of all 41 experimental spleens revealed that; (1) the standard deviation
at days 1-8 was significantly greater (P<0.01) than the standard deviation of all 41 experimental spleens (Appendix C); and (2) the standard deviation at day 17 was significantly less (P<0.01) than the standard deviation of all 41 experimental spleens (Appendix C).

Second, the standard deviation of the weight of 41 experimental spleens was significantly greater (P<0.01) than the standard deviation of the weight of 19 control spleens.

Third, spleens possessing a large cortex consisting of red pulp (erythropoietic tissue) and a smaller medullary area composed of white pulp (thrombopoietic tissue and lymphopoietic tissue) [Fig. 35, see also Part I, Fig. 19] (these spleens, henceforth, will be referred to as red pulp spleens because the majority of blood cells within them are erythrocytes) were heavier (P<0.001) than the spleens whose main cellular components were lymphocytes (Fig. 36) [henceforth referred to as white pulp spleens]. White pulp spleens were generally characterized by an increased number of lymphoblasts as well as increased red blood cell agglutination and lysis (Figs. 37, 38) compared to the red pulp spleens. Portions of erythrocytes were often observed in the cytoplasm of the macrophages of white pulp spleens (Fig. 39), but not red pulp spleens.

Rejection time of grafts of non-injected control newts

Complete rejection and the events leading to complete rejection of male allografts by male and female uninjected control newts did not differ significantly (Fig. 29) from the rejection phenomena associated with female allografts of uninjected control newts of both sexes.
Figure 29

Stages leading to and including complete allograft rejection. Mean number of days ± standard deviation in (Group 1) male allografts from non-treated control male and female newts; (Group 2) both male and female allografts from non-treated control male and female newts; (Group 3) female allografts from non-treated control male and female newts; and (Group 4) autographs from non-treated control male and female newts.
STAGES OF REJECTION

GROUP 1
GROUP 2
GROUP 3
GROUP 4

PRIMARY VASODILATION
RECIRCULATION
SIGNS OF REJECTION
COMPLETE REJECTION

DAYS (MEAN ± S.D.)

50
40
30
20
10

29
In all allografts examined, the mean times (in days) for the appearance of primary vasodilation (Figs. 40, 41 and 42), recirculation (Fig. 43), signs of rejection (Figs. 44, 45 and 46), and complete rejection (Fig. 47) were respectively $8.9 \pm 3.4$, $12.4 \pm 3.4$, $23.1 \pm 3.8$ and $40.6 \pm 7.2$ (Fig. 29). Similarly, there was no evidence of a sexually dimorphous response to skin allografts (Fig. 30).

**Rejection times of grafts of newts receiving PHA**

Sex of the newt or sex of the donor graft did not significantly alter ($P<0.05$) the times or variations of times at which primary vasodilation took place, recirculation was re-established, signs of rejection appeared, or complete rejection occurred (Appendix D, Tables 2 and 3). The rejection of male and female allografts by male and female newts was similar whether they received two injections of PHA (total of $1.70 \text{ mg of PHA at 0.85 mg per injection}$) or three injections (total of $2.55 \text{ mg of PHA at 0.85 mg per injection}$) [Appendix D, Tables 4 and 5].

The times (in days) for primary vasodilation, recirculation (Fig. 48), signs of rejection (Figs. 50, 52), and complete rejection (Fig. 54) in all allografts of newts receiving PHA prior to grafting were combined since the number of injections or the quantity of PHA injected had no apparent affect on the rejection phenomena and was found to be respectively $7.8 \pm 3.1$ (31 grafts), $12.2 \pm 2.5$ (32 grafts), $20.4 \pm 3.5$ (26 grafts), and $31.7 \pm 7.4$ (12 grafts) [Appendix D, Tables 6 and Fig. 31]. Male and female newts rejected allografts similarly.
Figure 30

Stages leading to and including complete allograft rejection. Mean number of days ± standard deviation in (Group 1) male and female allografts from non-treated control male newts; (Group 2) male and female allografts from non-treated control male and female newts; (Group 3) male and female allografts from non-treated control female newts.
DAYS (MEAN ± S.D.)

GROUP 1
GROUP 2
GROUP 3

STAGES OF REJECTION

PRIMARY
VASODILATION
RECIRCULATION
SIGNS OF
REJECTION
COMPLETE
REJECTION

STAGES OF REJECTION
Autographs remained viable as the allografts were rejected (Figs. 49, 51, 53, 55).

The mean times (in days) for the appearance of primary vasodilation (Figs. 56, 57, 58), recirculation (Fig. 59), signs of rejection (Fig. 60), and complete rejection (Fig. 61) for all allografts (all allograft data was combined since no sexual dimorphism was observed) from newts which received PHA after grafting were respectively 5.8 ± 1.3 (29 grafts), 9.1 ± 1.3 (17 grafts), 18.0 ± 3.1 (8 grafts) and 37.2 ± 5.0 (20 grafts), [Appendix D, Table 7 and Fig. 31].

With regard to the rejection of non-injected control allografts and allografts of newts receiving 0.1 ml sterile cold Ringers solution, the following sequence of events was detected; primary vasodilation at days 8.4 ± 3.6 (27 grafts), appearance of recirculation at days 11.3 ± 3.5 (27 grafts), signs of rejection at days 20.9 ± 5.0 (11 grafts), and complete rejection at days 36.1 ± 7.0 (42 grafts) [Appendix D, Table 8 and Fig. 31]. There was no evidence of a sexually dimorphous response to control allografts (Appendix D, Table 9) and no evidence of a significant difference in the way the two control groups rejected allografts.

The "t" test for uncorrelated means and "f" test revealed that there was no consistently significant difference (P<0.05) in the mean times or variations in times at which stages of allograft rejection appeared in control allografts and allografts of newts which had received PHA prior to grafting (Fig. 31). Although there was a significant difference (P<0.05) in times and variations in times at which primary vasodilation and recirculation occurred in control
Figure 31

Stages leading to and including complete allograft rejection.

Mean number of days ± standard deviation in (Group 1) control newts; (Group 2) newts which have received PHA prior to grafting; and (Group 3) newts which have received PHA after grafting.
DAYS (MEAN ± S.D.)

GROUP 1
GROUP 2
GROUP 3

PRIMARY VASODILATION
RECIRCULATION
SIGNS OF REJECTION
COMPLETE REJECTION

STAGES OF REJECTION
allografts and allografts of newts which received PHA after grafting (Fig. 31), this significant difference was not evident during the process of allograft rejection, and therefore, was not considered meaningful.

The toxic effects of PHA on newts

The response of newts all of which weighed from two to three grams to 1.7 mg and 2.6 mg of PHA-P was highly variable. During a 40-50 day period, approximately one-half of the animals receiving two and three injections of PHA died, many after the first injection of PHA. The newts which did survive were characterized by a decrease in muscular tone and appetite and a highly edemic dermis and epidermis. Erythema was often evident at the site of injection of PHA.
Histology of grafts

Sections of grafts from PHA-treated newts and from newts receiving either 0.1 ml of cold Ringers solution or no injections at all were similar throughout the various stages culminating in allograft rejection. This material thus served to verify the observations of the surface morphology of grafts (presented in the two preceding sections). The major characteristics (as determined by a histological examination of allografts as well as by correlation of the histology with surface morphology) of six different time periods leading to and including allografts rejection are described below.

1 to 2 days after grafting

Allografts and autografts appeared quite similar at this time. One or two cell layers of epidermis were usually observed covering the grafts in place for one day (Fig. 64). The epidermis was so loosely attached to the grafts that completely denuded areas occasionally were observed (Fig. 65). Cells of the skin glands located immediately subjacent to the epidermal basement membrane were undergoing the following alterations: (1) lysis of cell membranes (Fig. 66); (2) cytolytic alterations of the cytoplasm (Fig. 68); (3) rounding up and apical positioning of nuclei (Fig. 69) which would normally be elongated and located near the basal portion of the skin gland cells (Fig. 70); (4) an increase in the total number of nuclei (Fig. 71); and the number of pyknotic nuclei (Fig. 72) compared to that which was observed in skin glands at non-grafted sites (Fig. 67).

Viewed either from the surface (Fig. 73) or in cross-section
between the skin glands and the epidermal basement membranes possessed cytoplasmic processes. These processes were obvious because of the many cytoplasmic granules which they contained. There was no evidence of vascular invasion of the grafts and no accumulation of mononuclear cells in the dermis at this time.

4 to 5 days

Surface views and sections of grafts were quite variable in appearance when examined 4-5 days after grafting. The epidermis of some grafts was extremely hyperplastic, usually 4-5 cell layers thick (Fig. 75), while that of other grafts appeared normal (1-2 cell layers thick) [Fig. 77]. The surface views of the two grafts shown in figures 75 and 77 also reflected the difference in epidermal thickness. The processes of melanocytes were much more evident in the grafts with 1-2 cell layers of epidermis (Fig. 78) than the grafts with a hyperplastic epidermis (Fig. 76). Most of the melanocytes in the graft sites exhibited cytoplasmic processes (Figs. 76, 78), although the contour of a few of these cells appeared rounded in surface views (Fig. 78) as well as in cross-section (Fig. 79) due to the absence of granules in the cytoplasmic processes. Most of the skin glands resembled those observed at days 1-2 (Fig. 75). Large rounded nuclei enclosed by the basement membrane of the skin glands were observed occasionally (Fig. 80). There was little evidence of revascularization of the grafts at this time.
10 to 11 days

At ten and eleven days after grafting, the epidermis of a majority of the grafts was 1-2 cell layers thick (Fig. 81). The only area where hyperplastic epidermis existed was at the junction between the epidermis of the host and the graft (Fig. 82). Melanocytes observed at this time were generally rounded in appearance (Fig. 83), although a few did possess cytoplasmic processes. Many of the melanocytes were located in the dermis some distance from the epidermal basement membrane (Fig. 83). Nearly all of the glands of the allografts were in various stages of degeneration (as described in the previous section). However, there occasionally was evidence of glandular regeneration in the autografts (Fig. 82).

Primary vasodilation (Fig. 84) and recirculation (Fig. 81) of capillaries and arterioles of the dermis was observed in sections of the grafts. These sections corresponded to the surface views of primary vasodilation (Figs. 41, 42, 57 and 58) and recirculation (Figs. 43, 48, 51, 53, 55 and 59) discussed previously.

16 to 17 days

Sections of the 16-17 day autografts were similar to those of the grafts described in the 10 to 11 day section. Many melanocytes were located deep within the dermis and there were very few skin glands present. The blood supply to the grafts appeared normal with no lymphocytic infiltration of the autografts occurring. In contrast, control allografts and allografts from newts which had received PHA either before or after grafting began to demonstrate signs of rejection.
at approximately the sixteenth day. Secondary vasodilation was observed occasionally in sections (Fig. 85) and surface views (Fig. 60). In addition, lymphocytes began to accumulate in the graft beds of allografts (Figs. 86, 87). The accumulation of lymphocytes coincided with the onset of hemorrhage which was observed often dramatically in surface views of the grafts (Figs. 44, 45, 46, 50, 52). Very few skin glands were present in the 16 day old allografts (Figs. 86). Some melanocytes were located deep within the dermis, while others were seen near the basement membrane of the epidermis (Fig. 86).

23 to 24 days

Most of the allografts examined at days 23-24 were characterized by a moderate amount of lymphocytic infiltration. However, a few allografts were in the terminal stages of graft rejection (Fig. 88). Sections through the graft shown in Figure 88 revealed that there was an extensive cellular infiltration of the graft bed (Figs. 89, 90, 91). The same newt which was rejecting the allograft depicted in Figures 88, 89, 90 and 91 possessed a very healthy autograft (Fig. 92). Intact skin glands were observed occasionally in allografts during the final stages of graft rejection (Fig. 93).

41 to 46 days

Sections of all of the 41-46 day allografts were infiltrated extensively by lymphocytes (Fig. 94). The epidermis was being sloughed and the few melanocytes observed were located deep within the dermis
(Fig. 94). A surface view of the allograft shown in Figure 94 revealed that the melanocytes were small and rounded and blood was flowing through at least one part of the graft (Fig. 95). In contrast, forty-one and forty-six day autografts were quite healthy as indicated by sections (Fig. 96) and surface views (Fig. 97). The epidermis of the autografts was normal and melanocytes, although lacking processes were quite numerous (Fig. 97) as compared with allografts examined at similar times (Fig. 95). The skin glands of the autografts were abnormal in appearance, but did seem to be regenerating as indicated by the number of healthy cells positioned adjacent to the basement membrane (Fig. 96). The vascular supply to the autograft was quite similar to that of surrounding tissue (Fig. 97).
DISCUSSION

This study indicated that the intraperitoneal administration of PHA-P caused no consistent change in the morphology of the thymus, liver, lamina propria of the intestine and bone marrow of the adult newt. These findings are in agreement with the study of Elves et al., ('63), who found no changes in the liver of rats subsequent to the administration of PHA-M, but differs markedly from investigations showing alterations in the thymus and liver of mice (Machado and Lozio, '68), the liver of rabbits (Naspitz et al., '68) and the bone marrow of mice (Lozio et al., '69). The above-mentioned variations suggest that the species of animal used, the quantity and type of PHA (PHA-P or PHA-M) utilized and/or the timing and route of administration of PHA may determine the in vivo effects of PHA.

An investigation of the histology and weight of spleens from treated and untreated newts did, however, reveal two significant points. First, the variability of spleen weight in newts which received PHA was so much greater than the variability of spleen weights from untreated newts, that there was a significant difference in the standard deviations. Second, red pulp spleens (either from treated or untreated newts) were generally heavier than white pulp spleens (P<0.005) probably due to the greater number of cells in the red pulp spleens.
With regard to the first point, the variable spleen weights and cytoarchitecture of spleens of untreated newts noted in this study is similar to that seen in the study of Tooze and Davies (’68). The greater variability in the weights of spleens from PHA-treated newts suggests that the administration of PHA may have caused; (1) either abnormally high spleen weights by inducing lymphocyte blastogenesis; or (2) unusually low spleen weights by causing erythrocyte agglutination which would have led eventually to a depletion of the erythrocyte population of the spleen. The reason that erythrocyte agglutination was noted here and not in the study of Gamble (’66) was because in the present study the hemagglutinating activity of PHA was not removed by adsorption with erythrocytes prior to injection into the newts.

Although PHA did alter the cytoarchitecture of the spleen of the newt, it did not prolong the survival of skin allografts. Thus, it is impossible to theorize as to the origin of cells involved in the rejection of allografts. The findings concerning the effects of PHA on skin allografts survival are in agreement with the work of Elves (’66) and Kehn and Rigby (’67) who noted no prolongation of the survival of skin allografts of mice as a result of the administration of PHA. As was seen previously by Kehn and Rigby (’67), the present study confirmed that neither the dosage nor the timing of PHA administration with respect to the application of skin allografts affected rejection time.

In contrast to the observations made here, Markey et al., (’66) found that the injection of PHA into rabbits before grafting significantly increased the survival times of skin allografts and
St. Pierre et al., ('67) noted that the administration of PHA prior to grafting significantly increased graft survival times in mice, but that no increase was obtained when PHA was administered 24 hours after grafting. The variable observations of the studies cited above may be a result of the different species of animals and experimental procedures utilized.

The observations of this study further indicate that; (1) male and female allografts were rejected similarly whether from control newts or from newts which had received PHA before or after grafting and (2) that male newts, as had been previously observed (Erickson, '62; Quinn, '64; and Cohen, '66a, '66b), did not reject allografts differently from female newts.

The study of untreated allografts was essentially a re-investigation of a previous study (Cohen, '66a, '66b) on first-set skin allograft rejection in the newt, Diemictylus viridescens (now called Notopthalmus viridescens). However, because of the possible existence of a large number of naturally occurring inbred strains of newts in isolated areas and also because of the different techniques used in the present study, it was necessary to determine how closely the results of Cohen ('66a, '66b) coincided with results obtained from the group of newts utilized in this study. As a result of the study of allografts from treated as well as untreated newts, it was possible to extend the observations of Cohen ('66a, '66b).

Although the times of appearance of primary vasodilation, recirculation, signs of rejection and complete rejection were consistent with the time observed by Cohen ('66a, '66b), the variability as to the time of onset of these phenomena was significantly greater.
(P<0.01) in this study. Furthermore, in this study, complete cessation of blood flow through the allograft, rather than total melanocyte destruction, was utilized as the primary indication of complete allograft rejection. This may explain why complete rejection of allografts by control newts was judged to occur at 36.1 days in this study or 7.3 days later than was observed by Cohen ('66b). Melanocyte death was not utilized as the primary indicator of complete allograft rejection in this investigation for two reasons. First, melanocyte response to chlorobutanol (0.1% to 0.5% aqueous solution), caused by the migration of the cytoplasmic granules of melanocytes from a perinuclear position into the cytoplasmic processes, was barely perceptible (Figs. 62, 63) and in many cases could not be ascertained positively until after photographs of chlorobutanol-treated and non-treated grafts were available for comparison. Thus, melanocyte viability could seldom be determined immediately after observing the surface response of melanocytes to chlorobutanol. Secondly, melanocytes gradually migrated from a position immediately subjacent to the epidermal basement membrane into the deep dermis. This occurred in both allografts and autografts and was not a reliable indicator of the destruction of melanocytes. Once melanocytes had migrated into the deep dermis, they may have appeared brownish and small from the surface, but not necessarily due to death. Cohen ('66a, '66b) was able to illicit a more extensive expansion of melanocytes by treatment of the grafts with a 0.3% solution of chlorobutanol than was obtained in this investigation, but even in his study, the effects of chlorobutanol might very well have been altered by the change in position of the
melanocytes.

Since the morphological observations of allografts from PHA-treated and untreated newts were similar, all of the allografts were grouped in order to present a detailed histological sequence of events leading to and including complete allograft rejection. The observations made in this study are in agreement with those made previously by Cohen ("66a, '66b) with the one exception that hyperplasia of the epidermis covered first-set allografts. Cohen ('66a, '66b) saw no hyperplasia in the epidermis which covers first-set skin allografts, but in the present study hyperplastic epidermis was observed in the majority of allografts from both PHA-treated and untreated newts. This is in agreement with Sarkany ("66) and Sarkany and Caron ("66) who have demonstrated that intradermal injections of PHA had a mitogenic effect on guinea pig epidermal cells in vivo. Thus, our observations suggest that hyperplasia is always present over first-set skin allografts, but may be augmented by the administration of PHA.
SUMMARY

Sections and surface views of allografts and sections of thymuses, spleens, livers, intestines and bone marrows from control newts which received either intraperitoneal injections of 0.1 ml sterile cold Ringers solution or no injections at all were studied and compared with allografts and hemopoietic tissue of newts which received intraperitoneal injections of PHA either before or after grafting. PHA caused neither marked destruction of erythrocytes nor a blastogenic response of lymphocytes in the thymus, liver, intestine and bone marrow of the newt. However, the above-mentioned changes were observed in the spleen and there was also a significant difference in the standard deviations of the weights of spleens excised from PHA-treated and untreated newts. Red pulp spleens were generally heavier than white pulp spleens.

With regard to the rejection of control allografts, the following sequence of events was detected; primary vasodilation at days 8.4 ± 3.6, appearance of recirculation at days 11.3 ± 3.5, signs of rejection at days 20.9 ± 5.0 and complete rejection at days 36.1 ± 7.0. Neither the dosage of PHA, nor its administration in relation to the application of skin allografts significantly altered the times leading to and including complete rejection of allografts. All of the allografts studied demonstrated a wide range of variability in the events
culminating in complete graft rejection. There was no evidence of a sexually dimorphic response to any of the allografts examined.
PLATE V
EXPLANATION OF FIGURES

32. Male newt with male allograft (MA), female allograft (FA) and autograft (AU).

33. Apparatus for immobilizing newts and flattening grafts. Newts were placed in the apparatus so that the ventral surface of the newts and the grafts were pressed against the cover slip (CS).

34. Non-hemopoietic bone marrow (BM) of the femur of the newt. X 240

35. Cortex (SPC) and medulla (SPM) of a red pulp spleen. X 240

36. Lymphocytes (L) in a white pulp spleen. X 480

37. Lysis of erythrocyte cytoplasmic membranes (EL) indicated by the distorted shapes of the erythrocytes in a white pulp spleen. X 1700

38. Unphagocytized remains of erythrocytes (E) in a white pulp spleen. X 1700

39. Phagocytosis of erythrocytes by a splenic macrophage (SM) in a white pulp spleen. X 1700
PLATE VI
EXPLANATION OF FIGURES

40. Male allograft of a non-injected control male newt 8 days after grafting. No re-vascularization of the graft is evident at this time. X 50

41. Primary vasodilation (PV) in a male allograft of a non-injected control male newt four days after grafting. X 50

42. Primary vasodilation (PV) in a female allograft of a non-injected control male newt eight days after grafting. X 50

43. Recirculation (R) in a male allograft of a non-injected control male newt 11 days after grafting. X 50

44. Hemorrhage (H) in a female allograft of a non-injected control female newt 16 days after grafting. X 67

45. Hemorrhage (H) and secondary vasodilation (SV) in the same female allograft shown in figure 44, 18 days after grafting. X 67.

46. Hemorrhage (H) in a female allograft of a non-injected control female 23 days after grafting. X 50

47. Complete rejection in the same female allograft shown in figures 44 and 45, 30 days after grafting. Complete rejection is characterized mainly by a complete lack of blood flow through the graft. A small number of melanocytes (M) are evident. X 50
PLATE VII
EXPLANATION OF FIGURES

48. Recirculation (R) in a seven day female allograft of a female newt which received two injections of PHA prior to grafting. X 50

49. Seven day female autograft of a female newt which received two injections of PHA prior to grafting. X 50

50. Hemorrhage (H) and secondary vasodilation (SV) in the same allograft pictured in figure 48 at day 19. X 50

51. Recirculation (R) in the same female autograft pictured in figure 48 at day 19. X 50

52. Hemorrhage (H) and secondary vasodilation (SV) in the same female allograft shown in figures 48 and 50, 23 days after grafting. X 50

53. Recirculation (R) in the same female autograft shown in figures 49 and 51 at day 23. X 50

54. Complete rejection of the same female allograft depicted in figures 48, 50 and 52 at day 29. X 50

55. Recirculation (R) in the same female autograft depicted in figures 49, 51 and 53 at day 29. X 50
PLATE VIII
EXPLANATION OF FIGURES

56. No vascular invasion in an eight day female allograft of a male newt which received three injections of PHA after grafting. X 50

57. Primary vasodilation (PV) of a single blood vessel in a five day female allograft of a male newt which received three injections of PHA after grafting. X 50

58. Extensive primary vasodilation (PV) in a five day male allograft of a male newt which received three injections of PHA after grafting. X 50

59. Recirculation (R) in a 16 day male allograft of a male newt which received three injections of PHA after grafting. X 50

60. Secondary vasodilation (SV) and hemorrhage (H) in a 23 day female allograft of a female newt which received three injections of PHA after grafting. X 50

61. Nearly complete rejection in a 36 day female allograft of a female newt which received three injections of PHA after grafting. Blood flow is still evident in one area of the allograft (arrow). X 50

62. Male allograft of a PHA-treated (2 injections) male newt 15 days after grafting. Two melanocytes which will respond to chlorobutanol treatment are indicated by the arrows (see Fig. 63).

63. The same allograft that is shown in figure 62, 25 minutes after treatment with a 0.3% chlorobutanol solution. The melanocyte response to chlorobutanol is not dramatic, but is perceptible (arrows).
PLATE IX

EXPLANATION OF FIGURES

64. Epidermis (E) of a one day female allograft from a female non-injected control newt. X 480

65. Epidermal basement membrane (BM) of the same allograft shown in figure 64. X 480

66. Skin gland cells (arrow) in the dermis of a one day autograft from a female newt. The absence of cell membranes is apparent when comparing glands in this figure with glands located in a non-grafted site (figure 67). X 480

67. Cell membranes (CM) of non-grafted skin gland cells from a female newt 41 days after the last of the three PHA injections was administered. X 480

68. Pigment accumulation and cytolytic alterations (arrow) in a cell of a skin gland located in a one day autograft from a male newt which had received three injections of PHA prior to grafting. X 1700

69. Rounded up nuclei (N) located somewhat apically compared to a normal position (fig. 70) in a skin gland cell of a one day female allograft of a non-injected female newt. X 1700

70. Nucleus (N) at a normal position in a non-grafted skin gland of a control female newt. X 1700

71. Nuclei (arrow) [mostly of skin gland cells] enclosed by a basement membrane (BM) in a skin gland of a one day female allograft from a male newt. X 1700
PLATE X
EXPLANATION OF FIGURES

72. Pyknotic nuclei (PN) enclosed by a basement membrane (BM) of a skin gland located in a one day autograft from a male newt which received three injections of PHA prior to grafting. X 1700

73. Surface view demonstrating cytoplasmic process (CP) of melanocytes of a one day female allograft from a female newt. X 50

74. Cytoplasmic processes (CP) of a melanocyte in the same one day allograft shown in figure 73. X 1700

75. Hyperplastic epidermis (HE) [4-5 cell layers thick] of a 4 day male allograft from a male newt which had received three injections of PHA prior to grafting. Several skin glands (SG) are observed in the dermis. X 240

76. A surface view of the allograft shown in figure 75. Cytoplasmic processes (CP) of melanocytes are present, but the pattern of branching is not extensive. Re-vascularization of the graft is not evident. X 50

77. Epidermis (E) of a 4 day female allograft from a female newt. Note the ducts (D) of two skin glands opening to the surface. X 480

78. A surface view of the allograft shown in figure 77. Cytoplasmic processes (CP) of melanocytes and melanocytes (M) without cytoplasmic processes are evident. X 50

79. Melanocyte (M) with no cytoplasmic processes from a 4 day male allograft of a male control non-injected newt. X 1700
PLATE XI

EXPLANATION OF FIGURES

80. Large nuclei (N) enclosed by the basement membrane (BM) of a skin gland of a male allograft from a male newt which received three injections of PHA before grafting. X 1700

81. Epidermis (E) and dermal capillaries (CA) in an eleven day male allograft from a female newt which received three injections of PHA after grafting. X 480

82. Hyperplastic epidermis (HE) at the junction between the epidermis of a ten day autograft (AUE) [from a male newt which received 3 injections of PHA prior to grafting] and the host epidermis (HOE). Regenerating skin glands (RG) characterized by a group of cells positioned around a lumen are occasionally observed. X 240

83. Melanocytes (M) in the dermis of an 11 day male allograft from a male non-injected control newt. X 240

84. Primary vasodilation (PV) of capillaries (CA) in the dermis of a 10 day female allograft from a male newt which received 3 injections of PHA before grafting. X 480

85. Secondary vasodilation (SV) of blood vessels in the dermis of a 16 day female allograft from a male newt which received 3 injections of PHA before grafting. X 240

86. Lymphocytes (L) and melanocytes (M) in a 17 day male allograft from a male non-injected control newt. X 240

87. Lymphocytes (L) in the deep dermis of a 17 day male allograft from a male non-injected control newt. X 1700
PLATE XII
EXPLANATION OF FIGURES

88. A surface view of a 23 day female allograft in a terminal stage of rejection from a male newt which had received 3 injections of PHA after grafting. X 50

89. Large lymphocyte (LL), small lymphocyte (SL), and monocyte (MO) in the dermis of the same 23 day allograft shown in figure 88. X 480

90. Monocyte (MO) in the dermis of the same allograft shown in figures 88 and 89. X 1700

91. Small lymphocytes (SL) in the dermis of the same allograft shown in figures 88, 89 and 90. X 1700

92. Recirculation (R) through the 23 day autograft of the same newt which possessed the allograft shown in figures 88, 89, 90 and 91. X 50

93. Skin gland (SG) in the dermis of a 23 day allograft from a female newt which received 3 injections of PHA after grafting. X 480

94. Lymphocytes (L), epidermis (E) and melanocytes (M) in a 41 day male allograft from a non-injected control female newt. X 240

95. Melanocyte (M) and blood vessel (BV) in a surface view of the same allograft shown in figure 94. X 50

96. Epidermis (E) and regenerating skin gland (RG) of a 41 day autograft from a male non-injected control newt. X 240

97. Melanocytes (M) and blood vessel (BV) in a surface view of the same autograft shown in figure 96. X 50
APPENDIXES
APPENDIX A

PLASTIC EMBEDDING TECHNIQUE


2. Running water: 8-24 hours.

3. 70% ethanol: 2 changes over a 24 hour period.


5. Dehydrate: French squares are convenient containers for making these changes. The tissue may be left in 70% ethanol for an extended length of time.
   a. 70% ethanol: wash and leave in second change for 20 minutes.
   b. 95% ethanol: wash and leave in second change for 20 minutes.
   c. 100% ethanol: 3 changes at 15 minutes per change.
   d. Propylene oxide (room temperature): 3 changes at 15 minutes per change. An automatic dispenser is used to transfer alcohols and propylene oxide when a large number of tissues are processed. The changes of propylene oxide are made in a hood.

When dehydrating, a code number is etched on each bottle and the content of the bottle and the code
number are recorded on a separate sheet of paper because 100% ethanol and propylene oxide will cause any type of ink to become illegible.

6. The tissue is infiltrated with solution A at room temperature overnight.

Solution A: 5 ml hydroxyethanol methacrylate
1 ml 2-butoxyethanol (96% pure)
0.0169 gm benzoyl peroxide (explosive)

Benzoyl peroxide is mixed well on an electric mixer with hydroxyethanol methacrylate and 2-butoxyethanol until it is dissolved. Solution A is prepared immediately before it is used. It can be used for 3-4 days after it is initially prepared, if kept refrigerated at 0.0°C.

7. Method of embedding:

a. Horsecapsules (1/2 oz.) are placed in an upright position in a test tube rack (racks have tape attached lengthwise so that holes fit the capsules).

b. 6 ml of solution A and 3 drops of solution B are pipetted into each horsecapsule.

Solution B: 15 ml Carbo Wax 400
1 ml N,N-diemethlaniline
mix well

Solution B should be stored at room temperature for one month before use.

If more than 3 drops of solution B is added to 6 ml of solution A, the plastic will not polymerize.

c. Solution A and Solution B are stirred well.
d. The tissue is placed in the capsule with the surface to be cut, face down. The tissue and the capsule are inspected for the presence of air bubbles.

e. The capsule is covered with a lid and placed in an upright position in a test tube rack.

f. The rack is placed under a fluorescent light which aids in polymerization of the plastic.

g. Polymerization normally takes 2-3 hours.

8. Cutting technique:

a. The lid of the horsecapsule is removed and a thin layer of unpolymerized plastic is poured off.

b. The capsule is removed and the cutting surface of the plastic is made square using a straight saw and sculpturing tools. Some plastic is left around the tissue.

c. If the plastic block does not set up well, that portion of the plastic that has set up (if it contains the tissue) is removed and glued (epoxy glue) to a wooden block. If the plastic block does set up, the plastic block is placed in a chuck (machined so that it will fit into a rotating microtome). The chuck is designed so that it fits snugly around the plastic blocks.

d. A microtome blade stropped with a fine linen strop is used to cut 1-2μ sections. A pencil erasure occasionally is rubbed against the microtome blade in an effort to reduce static electricity.
e. A drop of 10% aqueous acetone is placed on a 22 mm cover slip. Plastic sections which are removed from the microtome blade with jewelers forceps are then placed shiny surface upward on the drop of 10% acetone. The acetone helps to remove wrinkles from the section. In addition, a small brush is utilized to flatten out sections and remove bubbles. The cover slip is held vertically so that the acetone and bubbles will run to the side of the cover slip.

f. The cover slips are placed on a warming table or in a 37°C. oven.

g. If the sections do not adhere to the cover slips during the staining procedure, adhesive tape is used to insure that sections remain on the cover slips.
APPENDIX B

HEMATOXYLIN AND EOSIN STAINING PROCEDURE FOR PLASTIC SECTIONS

1. Harris' hematoxylin: 30 minutes
2. Distilled water: wash
3. 1% hydrochloric acid in 70% ethanol: 1 dip
4. Tap water: wash
5. 0.3% ammonia water: until blue
6. Distilled water: wash
7. Distilled water: wash
8. 70% ethanol: 1 dip
9. 95% ethanol: 1 dip
10. Eosin in 95% ethanol: 5 minutes
    Eosin:
    0.1 gm erythrosin B
    0.05 gm orange G
    0.1 gm eosin y
    0.1 ml acetic acid
11. 95% ethanol: rinse
12. 95% ethanol: rinse
13. Isopropyl alcohol: rinse
14. Xylene
15. Xylene
### APPENDIX C

#### TABLE 1

<table>
<thead>
<tr>
<th>NUMBER AND TYPE OF SPLEENS EXAMINED</th>
<th>MEAN WT. (IN MG) ± STANDARD DEV.</th>
<th>SIGNIFICANT DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>41 Experimental Spleens</td>
<td>2.2 ± 2.2</td>
<td>S.D. p &lt; 0.01</td>
</tr>
<tr>
<td>19 Control Spleens</td>
<td>2.2 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>20 Male Experimental Spleens</td>
<td>2.4 ± 2.3</td>
<td>None</td>
</tr>
<tr>
<td>21 Female Experimental Spleens</td>
<td>1.8 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>13 Male Control Spleens</td>
<td>1.7 ± 0.9</td>
<td>Means</td>
</tr>
<tr>
<td>6 Female Control Spleens</td>
<td>2.9 ± 1.5</td>
<td>0.01 &lt; p &lt; 0.05</td>
</tr>
<tr>
<td>8 Red Pulp Experimental Spleens</td>
<td>5.7 ± 2.8</td>
<td>Means p &lt; 0.001</td>
</tr>
<tr>
<td>24 White Pulp Experimental Spleens</td>
<td>1.9 ± 2.8</td>
<td>S.D. p &lt; 0.01</td>
</tr>
<tr>
<td>5 Red Pulp Control Spleens</td>
<td>3.6 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>4 White Pulp Control Spleens</td>
<td>1.1 ± 0.5</td>
<td>Means</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.001 &lt; p &lt; 0.005</td>
</tr>
<tr>
<td>13 Control and Experimental Red Pulp Spleens</td>
<td>4.9 ± 2.5</td>
<td>Means p &lt; 0.001</td>
</tr>
<tr>
<td>28 Control and Experimental White Pulp Spleens</td>
<td>1.2 ± 0.5</td>
<td>S.D. p &lt; 0.05</td>
</tr>
<tr>
<td>9 Experimental Spleens Excised</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-8 Days After Final Inj. of PHA</td>
<td>3.6 ± 3.7</td>
<td>S.D.</td>
</tr>
<tr>
<td>41 Experimental Spleens</td>
<td>2.2 ± 2.2</td>
<td>0.01 &lt; p &lt; 0.05</td>
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<td></td>
</tr>
<tr>
<td>9-14 Days After Final Inj. of PHA</td>
<td>2.2 ± 2.1</td>
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<td></td>
</tr>
<tr>
<td>4 Experimental Spleens Excised</td>
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<td></td>
</tr>
<tr>
<td>17 Days After Final Inj. of PHA</td>
<td>1.1 ± 0.4</td>
<td>S.D.</td>
</tr>
<tr>
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<td>0.01 &lt; p &lt; 0.05</td>
</tr>
<tr>
<td>6 Experimental Spleens Excised</td>
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<tr>
<td>23 Days After Final Inj. of PHA</td>
<td>2.4 ± 1.1</td>
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</tr>
<tr>
<td>9 Experimental Spleens Excised</td>
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</tr>
<tr>
<td>39-49 Days After Final Inj. of PHA</td>
<td>1.5 ± 1.6</td>
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APPENDIX D

ALLOGRAFTS FROM NEWTS WHICH RECEIVED TWO INJECTIONS OF PHA PRIOR TO GRAFTING

TABLE 2

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<tr>
<th>EVENT</th>
<th>ALLOGRAFT</th>
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<tbody>
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<td>Male</td>
<td>9</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Male and Female</td>
<td>19</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td>Recirculation</td>
<td>Male</td>
<td>10</td>
<td>12.5 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11</td>
<td>10.8 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Male and Female</td>
<td>21</td>
<td>11.6 ± 2.2</td>
</tr>
<tr>
<td>Signs of Rejection</td>
<td>Male</td>
<td>8</td>
<td>19.6 ± 2.6</td>
</tr>
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<td></td>
<td>Female</td>
<td>10</td>
<td>19.3 ± 4.7</td>
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<tr>
<td></td>
<td>Male and Female</td>
<td>18</td>
<td>19.4 ± 3.8</td>
</tr>
<tr>
<td>Complete Rejection</td>
<td>Male</td>
<td>5</td>
<td>26.4 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3</td>
<td>30.0 ± 4.6</td>
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TABLE 3

<table>
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<th>MEAN ± S.D</th>
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<td>7.9 ± 1.9</td>
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<td>Female</td>
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<td>6.8 ± 1.7</td>
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<td></td>
<td>Male and Female</td>
<td>19</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td>Recirculation</td>
<td>Male</td>
<td>11</td>
<td>11.8 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10</td>
<td>11.4 ± 2.2</td>
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<td></td>
<td>Male and Female</td>
<td>21</td>
<td>11.6 ± 2.2</td>
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<td>9</td>
<td>21.4 ± 2.2</td>
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<td></td>
<td>Female</td>
<td>9</td>
<td>17.4 ± 2.1</td>
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<td>Male and Female</td>
<td>18</td>
<td>19.4 ± 3.8</td>
</tr>
<tr>
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<td>Male</td>
<td>4</td>
<td>29.0 ± 4.9</td>
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<td></td>
<td>Female</td>
<td>4</td>
<td>26.5 ± 2.6</td>
</tr>
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<td>Male and Female</td>
<td>8</td>
<td>27.8 ± 3.9</td>
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</thead>
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<td>9.6 ± 4.7</td>
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<td>Female</td>
<td>3</td>
<td>6.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Male and Female</td>
<td>12</td>
<td>8.7 ± 4.4</td>
</tr>
<tr>
<td>Recirculation</td>
<td>Male</td>
<td>9</td>
<td>13.7 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>2</td>
<td>12.0 ± 4.2</td>
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<td></td>
<td>Male and Female</td>
<td>11</td>
<td>13.4 ± 2.9</td>
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<td>Signs of Rejection</td>
<td>Male</td>
<td>6</td>
<td>22.5 ± 0.5</td>
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<td></td>
<td>Female</td>
<td>2</td>
<td>22.5 ± 0.7</td>
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<td>Male and Female</td>
<td>8</td>
<td>22.5 ± 0.5</td>
</tr>
<tr>
<td>Complete Rejection</td>
<td>Male</td>
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<td>39.5 ± 6.4</td>
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<tr>
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<td>Female</td>
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Table 5

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<td>Female</td>
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<td>6.0 ± 1.6</td>
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<td></td>
<td>Male and Female</td>
<td>12</td>
<td>8.7 ± 4.4</td>
</tr>
<tr>
<td>Recirculation</td>
<td>Male</td>
<td>5</td>
<td>14.0 ± 1.0</td>
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<tr>
<td></td>
<td>Female</td>
<td>6</td>
<td>12.8 ± 3.9</td>
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<tr>
<td></td>
<td>Male and Female</td>
<td>11</td>
<td>13.4 ± 2.9</td>
</tr>
<tr>
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<td>Male</td>
<td>2</td>
<td>22.5 ± 0.7</td>
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<tr>
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<td>Female</td>
<td>2</td>
<td>22.5 ± 0.5</td>
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<tr>
<td></td>
<td>Male and Female</td>
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<td>22.5 ± 0.5</td>
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<tr>
<td>Complete Rejection</td>
<td>Male</td>
<td>2</td>
<td>37.0 ± 4.2</td>
</tr>
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<td></td>
<td>Female</td>
<td>2</td>
<td>37.0 ± 4.2</td>
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### ALL ALLOGRAFTS FROM NEWTS WHICH RECEIVED PHA BEFORE GRAFTING

**TABLE 6**

<table>
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<td>7.8 ± 3.1</td>
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<tr>
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<td>12.2 ± 2.5</td>
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<tr>
<td>Signs of Rejection</td>
<td>Male and Female</td>
<td>26</td>
<td>20.4 ± 3.5</td>
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<td>Male and Female</td>
<td>12</td>
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### ALL ALLOGRAFTS FROM NEWTS WHICH RECEIVED PHA AFTER GRAFTING

**TABLE 7**

<table>
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<th>MEAN ± S.D.</th>
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<td>5.8 ± 1.3</td>
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<td>Male and Female</td>
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<td>9.1 ± 1.3</td>
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<tr>
<td>Signs of Rejection</td>
<td>Male and Female</td>
<td>8</td>
<td>18.0 ± 3.1</td>
</tr>
<tr>
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<td>Male and Female</td>
<td>20</td>
<td>37.2 ± 5.0</td>
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### CONTROL ALLOGRAFTS FROM NON-INJECTED CONTROL NEWTS AND NEWTS RECEIVING 0.1 ML COLD RINGERS'

**TABLE 8**

<table>
<thead>
<tr>
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<th>ALLOGRAFT</th>
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<th>MEAN ± S.D.</th>
</tr>
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<tbody>
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<td>Primary Vasodilation</td>
<td>Male and Female</td>
<td>27</td>
<td>8.4 ± 3.6</td>
</tr>
<tr>
<td>Recirculation</td>
<td>Male and Female</td>
<td>27</td>
<td>11.3 ± 3.5</td>
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<tr>
<td>Signs of Rejection</td>
<td>Male and Female</td>
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<td>20.9 ± 5.0</td>
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<td>Male and Female</td>
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<td>36.1 ± 7.0</td>
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CONTROL ALLOGRAFT FROM NON-INJECTED CONTROL NEWTS AND NEWTS RECEIVING 0.1 ML COLD RINGERS’

TABLE 9

<table>
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<th>NUMBER</th>
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<tbody>
<tr>
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<td>Male and Female</td>
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<td>8.4 ± 3.6</td>
</tr>
<tr>
<td>Recirculation</td>
<td>Male and Female</td>
<td>26</td>
<td>10.8 ± 3.4</td>
</tr>
<tr>
<td>Signs of Rejection</td>
<td>Male and Female</td>
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<td>20.7 ± 4.6</td>
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<td>Male and Female</td>
<td>40</td>
<td>35.8 ± 7.1</td>
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</tbody>
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BIBLIOGRAPHY


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