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LYMPHOPOIESIS AND LYMPH NODE HISTOGENESIS
IN THE EMBRYONIC AND NEONATAL RABBIT.

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LYMPHOPOIESIS AND LYMPH NODE HISTOGENESIS IN THE
EMBRYONIC AND NEONATAL RABBIT

DISSERTATION

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

By

Jeptha Ray Hostetler, B.A.

* * * * *

The Ohio State University
1967

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CONTENTS

	Page
ACKNOWLEDGMENTS	ii
VITA	iii
ILLUSTRATIONS	v
INTRODUCTION	1
MATERIALS AND METHODS	5
OBSERVATIONS	8
Development of the Lumbar (Para-Aortic)	
Lymph Nodes	9
Development of the Popliteal Lymph Nodes	14
Thymus	15
Spleen	16
Appendix	16
DISCUSSION	18
APPENDIXES	34
BIBLIOGRAPHY	42

ILLUSTRATIONS

Figure	Page
1. Cross section of the abdominal aorta with adjacent lymphatic network--17-day rabbit embryo.....	24
2. Mesenchymal condensation--lumbar lymph node anlage of a 18-day rabbit embryo.....	24
3. A 19-day rabbit embryo--lumbar lymph node anlage.....	24
4. A 19-day rabbit embryo--lumbar lymph node anlage.....	24
5. Lumbar lymph node anlage from a 19-day rabbit embryo.....	26
6. A 19-day rabbit embryo--lumbar lymph node anlage.....	26
7. A 21-day rabbit embryo--lumbar lymph node....	26
8. A 21-day rabbit embryo--lumbar lymph node....	26
9. Lumbar lymph node from a 21-day rabbit embryo.....	28
10. Lumbar lymph node from a 21-day rabbit embryo.....	28
11. A 22-day rabbit embryo--lumbar lymph node....	28
12. A 22-day rabbit embryo--lumbar lymph node....	28
13. Lumbar lymph node from a 23-day rabbit embryo.....	30
14. Lumbar lymph node from a 23-day rabbit embryo.....	30
15. A 24-day rabbit embryo--lumbar lymph node showing lymphocytes in small blood vessel.....	30

Figure		Page
16.	A 25-day rabbit embryo--lumbar lymph node showing lymphocyte in vessel wall.....	30
17.	Post-capillary venule from a lumbar lymph node of a 6-week-old rabbit.....	32
18.	Lumbar lymph node from a 27-day rabbit embryo--erythropoietic focus in medulla....	32
19.	A 25-day rabbit embryo--spleen.....	32
20.	A 19-day rabbit embryo--thymus.....	32

INTRODUCTION

The lymphocyte has been shown not only to play an intimate role in the immune response (Craddock et al., '49; Gowans, '62; Shoenberg et al., '63; McFarland and Heilman, '65; McFarland et al., '66) and inflammatory reaction (Downey, '22; Holub, '62; Wulff, '62, '63), but also to act as a circulating stem cell under the proper environmental conditions (Carstairs, '62; Gowans, '62; Truman and Cooper, '63; Tanaka et al., '63; Ling and Soothill, '64; Block, '64; Parrot and DeSousa, '66; Chesson et al., '66; Hannon and Bussard, '66; Speirs, '67; Bennett and Cudkowicz, '67). Morphological, histochemical, and experimental studies have resulted in considerable changes in the concepts regarding the possible mode or modes of origin, pathways of differentiation, cellular inter-relationships, and the functional capabilities of the lymphocyte and lymphocytic precursors.

Various experimental studies have indicated the existence of at least two populations of lymphocytes (Caffrey et al., '62; Everett et al., '64), have shown that lymphocytes undergo continual recirculation (Fichtelius, '59; Gowans, '59; Rankin, '60; Fichtelius et al., '61; Gowans and Knight, '64) and have suggested

that lymphocytes formed in the thymus during the embryonic period are disseminated to other organs (Harris and Ford, '63; Murray and Woods, '64A, '64B; Törő and Oláh, '67) where they give rise to the lymphocytic cells which comprise these tissues. At the present time considerable doubt and speculation exists as to the mode of origin and source of lymphocytes in both the adult and fetal tissues and organs.

The older concepts indicating the mesenchymal derivation of lymphocytes and lymphocytic precursors have been questioned, primarily in regard to the origin and differentiation of lymphocytes during the embryonic and neonatal periods. It generally has been regarded that stellate reticular or mesenchymal cells contribute directly to the lymphocyte pool by transforming into lymphoblasts (Danchakoff, '15, 16A, 16B; Han, '61) or into medium-sized lymphocytes (Saxer, 1896; Sabin, '04-05; Lewis, '09; Downey and Weidenreich, '12; Maximow, '09, '27); Theil and Downey, '21; Rothermel, '29; Ackerman, '66, '67A). This concept has been modified recently by studies performed on the embryonic mammalian and avian thymus and the avian bursa of Fabricius which suggest that cells of the lymphocytic series also may be derived from undifferentiated endodermal-epithelial cells during fetal development (Ackerman and Knouff, '59; Ackerman, '62; Ackerman and Knouff, '63, '64, '65). An epithelial

origin of lymphocytes in the lymphocytic nodules of the gastrointestinal tract also has been proposed (Sutherland et al., '64; Archer et al., '64, '64B), but with somewhat less experimental and morphological support. The concept of an epithelial origin of lymphocytic cells in the bursa of Fabricius and in the intestinal tract has been challenged by experimental studies employing autoradiographic techniques and chromosome markers (Moore and Owen, '66). These investigations have indicated that stem cells possibly derived from the hemopoietic cells of the yolk sac (Moore and Owen, '67) migrate into and proliferate in Peyer's patches, spleen, bone marrow, and bursa (Evans et al., '67). In addition, investigations of the intestinal tract of mammalian embryos have shown that there is no morphological evidence for the epithelial contribution to lymphopoiesis in the gastrointestinal tract, but rather, lymphocytic cells may arise from the in situ transformation of reticular or mesenchymal cells and are complemented by a second population of blood-borne lymphocytes (Ackerman, '66; '67A). Although the transformation of precursor cells into lymphocytic elements is of major importance during the embryonic and neonatal periods of development, the homoplastic proliferation, dissemination, and/or recirculation of lymphocytes may be the principal means of maintaining the lymphocyte

population in the definitive lymphocytic tissues and organs.

The purpose of this investigation is to examine critically the mode of origin and differentiation of the first cells of the lymphocytic series appearing in the lymph nodes of embryonic and neonatal rabbits. In light of the concept of lymphocyte dissemination from the thymus in embryonic and neonatal mammals, it is necessary to determine the sequential order of the production of lymphocytes in the thymus as well as other lymphocytic organs, i.e., lymph nodes, spleen, and appendix in the embryonic and neonatal rabbit.

In order to study embryonic and neonatal material the present investigation utilizes refinements in histological techniques which produce little or no cellular or tissue distortion. By using tissues fixed with the newer fixatives employed in electron microscopy, embedding the tissues in epoxy resin and examining thin (1 micron) sections of the plastic embedded material, it is possible to study cellular morphology and relationships with a precision not possible with older routine techniques.

MATERIALS AND METHODS

New Zealand white rabbits were used in this investigation. The gestation age was counted from the day following breeding as the first day of gestation; the day following birth was considered neonatal day one. Two pregnant does were sacrificed on each day of gestation from the seventeenth to the thirty-first day. Lumbar (para-aortic) and popliteal lymph nodes, thymus, spleen, and appendix were obtained from a total of 264 embryos from 17-31 days of gestation. In addition, lymphocytic tissues were removed from 20 neonatal rabbits from day one to day eighteen, from one 6-week-old rabbit and from two adult rabbits. Lumbar lymph nodes were obtained from embryos by removing and sectioning the entire abdominal aorta with its surrounding connective tissue and fat.

Whole embryos from 17-23 days of gestation were selected and fixed in one of three different fixatives: formalin-sublimate-acetic acid for 24 hours, formal-Zenker's solution for 24 hours, and 10% aqueous acrolein for 3 to 24 hours. These whole embryos were dehydrated through ethanol, processed through graded parlodion and embedded in paraffin (Appendix F). Serial transverse, sagittal, and frontal sections were obtained on entire embryos.

Alternate serial sections from whole embryos were stained with one of the following four procedures: 0.5% aqueous toluidine blue, hematoxylin and eosin, periodic acid-Schiff's reaction, and the periodic acid-Schiff's-hematoxylin-orange G combination. Sections were observed with phase and light microscopy to determine the various areas of the body where lymph node development occurs, to determine the general pattern of the development of lymphocytic tissues and organs in the rabbit, and to correlate these sections with the plastic embedded material.

For detailed cytological studies, lymphocytic tissues were removed from the rabbits and fixed in two different fixatives: cold 2.5% gluteraldehyde in 0.1 cacodylate buffer (Appendix D) (pH 7.2) containing 1% sucrose for 24 hours and 10% Acrolein (Appendix D) for thirty minutes to three hours. These tissues were subsequently cut into approximately one cubic millimeter blocks and post-fixed in 1% osmium tetroxide for one hour. Tissues were dehydrated in ethanol and embedded in either Araldite 502 or Epon 812 (Appendix A, B, C; Luft, '61).

Plastic embedded tissues, sectioned at one micron on the Porter-Blum ultra-microtome, were floated out on 10% acetone and affixed to glass slides by allowing the sections to dry at 60-70° C. for 3-5 minutes. These sections were stained with either 2% basic fuchsin

(Appendix E) in 50% acetone for 20-30 minutes at 60° C., or with 1% aqueous methylene blue (Appendix G; Delbert and Phillipott, '67) for one minute at 90-100° C. Plastic sections were observed with both light and phase contrast microscopy to determine the pathway of lymphocyte development and/or appearance in the lymphocytic tissues of the embryonic and neonatal rabbit.

OBSERVATIONS

Lymph node anlage develop almost simultaneously in the embryonic rabbit. These anlagen are represented either by the formation of anastomosing networks of lymphatic vessels (Figure 1) or by large lymphatic sacs which are found in close association with major vascular channels. The first embryonic lymph node anlage include the jugular lymph sac, axillary, submandibular, para-aortic, and popliteal plexuses. Examination of serial sections of whole embryos between 17 and 23 days of gestation indicates that most of the major lymph node primordia are present by the eighteenth day of embryonic development.

As indicated, the earliest evidence of lymph node development results from the joining of lymphatic vessels in an anastomosing plexus which is closely associated with an adjacent blood vessel. As the network of lymphatic vessels form, the mesenchymal tissue remaining between these channels forms connective tissue bridges or trabeculae. As these lymphatic vessels increase in size there occurs a further condensation of mesenchymal tissue within the bridges, in areas immediately surrounding the lymphatic network and along one or more walls of the

lymphatic vessels (Figure 2). Vascularization, evidenced by the invasion of capillaries into the primitive lymph node, soon occurs. Further growth and development of the lymph node appears largely due to the mitosis and proliferation of mesenchymal or reticular cells.

At 19-20 days of gestation, lymphocytes of small and medium-size make their first appearance, approximately 24 hours after the condensation of mesenchymal cells in the developing node. The number of these lymphocytes per given area of lymph node remains constant for several days. There appears to be a rapid increase in the number of lymphocytic cells in the developing nodes after the twenty-fifth day of gestation. As the number of lymphocytes increase the cortex and medulla soon become delineated. The majority of lymph nodes formed during the prenatal period have rounded or oval contours while others form extended chains, e.g., those along the abdominal aorta and carotid arteries. Early lymphocytic nodule formation is seen 2-3 days prior to birth, although germinal centers do not develop until 2-3 weeks postnatally.

Development of the lumbar (para-aortic) lymph nodes. By the seventeenth day of embryonic development, anastomosing networks of endothelial-lined lymphatic vessels lie lateral to the abdominal aorta, extending between the renal arteries and the bifurcation of the

aorta (Figure 1). Serial sections indicate that small lymphatic channels, which begin either blindly or as flattened tissue spaces, empty into the larger lymphatic vessels lying parallel to the aorta. By the eighteenth day of gestation there is an apparent widening of the vessels comprising the lymphatic network of the lymph node anlage with a concomitant condensation of mesenchymal cells directly related to the development of the node (Figure 2, 3). At this time small arteries and capillaries enter areas of mesenchymal condensation in the lymph node primordia. Lymphocytes and lymphoblasts are not present in the primitive nodes of the lumbar region at this stage of development.

As development progresses, lymphocytes gradually make their appearance in the lymph node anlage (Figure 4). These cells appear initially on the nineteenth day of gestation and are randomly distributed within the stroma of the node with no apparent association or clustering near the small blood vessels. No lymphoblasts are seen. Small and medium-sized lymphocytes are the first cells of the lymphocytic series present in the lumbar lymph nodes. Cells considered to be transitional between stellate mesenchymal cells and medium-sized lymphocytes can be recognized in these developing nodes (Figures 5-14). These cells assume a rounded cellular and nuclear contour

with a thickening of the nuclear membrane, a condensation of chromatin and a gradual increase of cytoplasmic basophilia. There is a progressive decrease in cell size.

The population of lymphocytes remains relatively constant and sparse as the lumbar lymph nodes continue to enlarge. The number of cells exhibiting morphological characteristics intermediate between stellate mesenchymal cells and medium-sized lymphocytes tends to increase through the twenty-fourth day of gestation. There is an increase in cellularity in the more cranial regions of the individual lymph nodes beginning on the twenty-fourth day as a result of the proliferation of both reticular and lymphocytic cells. This regional change in the cellular population within the developing lymph node marks the earliest signs of the sub-division of the lymph node into a primitive cortex and medulla. A distinct regional development of the cortex and medulla is evident by the twenty-seventh embryonic day. During the histogenesis of an individual lumbar node the lymphatic vessels comprising the original lymphatic network appear to give rise to the cortical, medullary, and subcapsular sinuses. By the twenty-seventh day of gestation the subcapsular sinus is well developed and tends to encompass the node. Reticular or reticular-endothelial cells span or bridge the subcapsular and medullary sinuses by the twenty-ninth

day of gestation. A delicate connective tissue capsule begins to form around the lymph node several days prior to birth. This capsule thickens, and completely surrounds the node by the end of the first postnatal week.

As the cortical and medullary regions of the lumbar lymph nodes begin to form on the twenty-fourth day of gestation, a moderate number of lymphocytes of both small and medium size are found in the medullary sinuses. A few lymphocytes also are found at this time in the small arteries and veins located in the cortex and medulla (Figure 15). In several instances a lymphocyte has been noted in both the small veins and developing lymphatic sinuses as early as the twenty-first day of gestation (Figure 10). Although not observed prior to the twenty-fourth day of embryonic development an occasional lymphocyte has been found within the walls of the blood vessels and sinuses at this stage of lymph node development (Figure 16). The number of lymphocytes present within the walls of these vessels increases during the later part of embryonic development and during the neonatal period (Figure 17). These lymphocytes appear to pass through and/or between the endothelial cells lining the blood vessels and although the direction of movement can not be precisely determined in histological sections, the morphological features established by these cells and knowledge of the pattern of

lymphocyte motility suggest that they probably pass from the blood vascular system into the parenchyma of the lymph nodes. It is not until after lymphocytes are observed in the small blood vessels of the developing lymph nodes that there is a rapid increase in the number of lymphocytes present within the stroma. This increase in numbers of lymphocytes is quite apparent by the twenty-seventh day of gestation and continues until the cortex becomes densely packed with lymphocytes and lymphocytic nodules begin to form just prior to birth.

Lymphoblasts do not appear in the lumbar nodes until after 27 days of gestation. These large, round, deeply basophilic cells may arise by the direct transformation of the stellate reticular or mesenchymal cells in the cortical area, since morphological transitions between these cells can be distinguished. Lymphoblasts undergoing subsequent differentiation and maturation appear to contribute to the lymphocyte population present in the lymph node.

Erythropoietic and granulopoietic activity is noted in the lumbar and popliteal lymph nodes after their lymphocytic nature has been established. These activities are transitory with the erythropoietic phase taking place between 24-27 days of gestation. Developing erythrocytic cells are formed in localized areas within the stroma of

both the cortex and medulla; a moderate number of mature erythrocytes and normoblasts also are found in the medullary sinuses (Figure 18). Unlike the localized clusters of erythrocytic elements, neutrophilic myelocytes are scattered randomly throughout the developing node from 25-27 embryonic days.

Development of the popliteal lymph nodes. The formation and development of the popliteal lymph nodes is similar in nearly every respect to that observed in the lymph nodes of the lumbar chain. Small and medium-sized lymphocytes appear in the popliteal node primordia on the nineteenth embryonic day and represent the initial lymphocytic cells to be seen within the nodes. As in the lumbar nodes, transitional forms intermediate between mesenchymal cells and medium-sized lymphocytes may be distinguished. After the primary lymphocyte population is established in the popliteal lymph nodes the growth and development of these nodes precedes one to two days more rapidly than that described for the lumbar nodes. There is a rapid increase in lymphocytes within the popliteal nodes beginning on the twenty-fifth day of embryonic development; lymphoblasts appear on the twenty-seventh day of gestation. Transitory granulopoietic and erythropoietic activity is evident from the twenty-fourth to twenty-seventh embryonic day. The organizational pattern of the popliteal

lymph nodes differs from that of the lumbar nodes in that the popliteal nodes assume a more rounded contour and the cortical region of the node tends to completely surround the medulla. The lymphocytic nodule formation begins two to three days prior to birth although germinal centers do not develop until two to three weeks postnatally.

Thymus. At 17 days of gestation the thymus of the embryonic rabbit is almost entirely epithelial except for several rare lymphoblasts which tend to be located within the more central regions of the thymic lobules. By the eighteenth embryonic day lymphoblasts are present in moderate numbers. At this time a few small and medium-sized lymphocytes also are seen scattered within the lobules of the thymus. Cells, which are considered to be transitional between undifferentiated epithelial cells and medium-sized lymphocytes, are encountered frequently beginning on the eighteenth embryonic day (Figure 20). During the seventeenth and eighteenth days of gestation cells of the granulocytic series are present in the connective tissue adjacent to and between thymic lobules. As the thymus continues to develop, lymphocytes increase rapidly in number and by the twenty-first day small and medium lymphocytes may be seen in connective tissue around thymic lobules. Lymphocytes can also be found in lymphatic vessels and small veins present in the connective tissue

region near the thymus. Vascularization of the thymus does not begin until 20-21 days of gestation.

Spleen. The histogenesis of the spleen begins as a mesenchymal condensation within the greater omentum on the twenty to twenty-first day of gestation. The subsequent growth and proliferation of these mesenchymal elements results in a gradual enlargement and evagination of the developing spleen. The splenic primordium remains attached to the omentum at the hilar region, where the splenic artery and vein enter. Hemopoiesis begins in the spleen on the twenty-third embryonic day as isolated foci of erythropoietic activity. On the twenty-fifth day of gestation the first cells of the lymphocytic series make their appearance in the form of small and medium-sized lymphocytes which are localized in areas directly surrounding the small follicular arteries (Figure 19). These cells increase rapidly in number between 25-29 days of gestation resulting in a histological picture similar to that observed in the definitive spleen. Preliminary observations suggest that, as in lymph node development, the initial lymphocytic cells arise from the transformation of mesenchymal cells.

Appendix. Small and medium-sized lymphocytes represent the first lymphocytic cells evident within the appendix of the rabbit. These cells are found within the

lamina propria on the twenty-eighth to twenty-ninth day of gestation. These lymphocytes appear to develop in situ from mesenchymal or reticular cells in this region.

Morphological evidence suggesting the infiltration of lymphocytes from the blood vascular system into the appendix is seen several days later, i.e., two to three days after birth. Critical evaluation of the epithelium lining the villi and glands of the developing appendix reveals no evidence for an epithelial contribution to lymphopoiesis in the appendix of the rabbit during prenatal and postnatal development.

DISCUSSION

The present investigation has shown a definite sequential order for the appearance of lymphocytes in developing lymphocytic tissues and organs in the fetal rabbit. The thymus represents the first lymphocytic organ to develop in the embryonic rabbit, beginning at 17 days of gestation, followed at 19 days by the lymph nodes, and at 25 days by the spleen, and finally by the appendix just prior to birth. In addition, small and medium-sized lymphocytes are the first lymphocytic cells to appear in these organs and tissues, with the exception of the thymus where lymphoblastic transformation precedes the appearance of lymphocytes. It should be noted that cells of the lymphocytic series are evident in these organs 1-2 days earlier than has been previously reported (Archer et al., '64B; Hostetler and Ackerman, '66; Sutherland et al., '64). The use of the newer techniques of tissue preservation and histological preparation coupled with phase contrast microscopy has not only greatly facilitated the recognition of lymphocytes in developing lymphocytic tissues, but has permitted the more precise identification of the cytological changes which occur during the process of lymphopoiesis.

Evidence presented in this study regarding the mode of origin and pattern of differentiation of the lymphocyte in the lymph nodes during embryogenesis indicates that the first lymphocytic elements found in the lymph node arise in situ by the transformation of stromal mesenchymal or reticular cells. Cellular modulations indicating the transformation of mesenchymal cells into medium-sized lymphocytes were frequently encountered in areas of mesenchymal condensation in the primitive node and later in the cortex and medulla. This initial population of lymphocytes remains sparse and constant until several days prior to birth when there is a rapid increase in the number of lymphocytes within the node. Coincident with this increase, clusters of lymphocytes can be found to localize around and near the blood vessels in the lymph node. Both small and medium-sized lymphocytes may be seen within the lumina and in the walls of the smaller vessels. These observations suggest that lymphocytes present in the blood stream pass through the small blood vessels and enter the developing lymph node during the later stages of fetal development. These findings are consistent with the reports of other investigators who have shown that lymphocytes enter the definitive lymph nodes and other lymphocytic organs via small veins and post-capillary venules (Gowans, '64; Dawson and Masur,

'29; Smith and Henon, '59; Marchesi and Gowans, '64; Gowans and Knight, '64).

In contrast to the thymus, the first lymphocytic cells found in the lymph nodes during embryogenesis are medium-sized, light to moderately basophilic lymphocytes rather than large, deeply basophilic lymphoblasts. A similar condition exists in the embryonic spleen and appendix. The presence of lymphocytes in lymph nodes and other non-thymic lymphocytic organs of the rabbit prior to the appearance of lymphoblasts is consistent with the possibility of the dissemination of lymphocytes from the thymus via the blood stream and the infiltration of these cells into other potential lymphocytic organs and tissues. The interpretation of our morphological observations suggests strongly that many of the lymphocytes found within the developing lymph node during fetal life do arise in situ by the direct transformation of stromal reticular or mesenchymal cells. These observations suggest that lymphocytes derived from these stromal elements of the lymph node comprise the primary population of lymphocytes within the node. This population of lymphocytes soon is augmented by a second population of lymphocytes which enter the lymph node by infiltrating from the vascular system. Perhaps, after the contribution of reticular or mesenchymal cells to the lymphocyte population during the

fetal life, subsequent reticular cell transformation along this pathway is no longer possible (Rieke et al., '63) or represents only a minor contribution of lymphopoiesis after birth.

The in situ differentiation of lymphocytes from mesenchymal cells in the developing lymph node, followed by the infiltration of lymphocytes from the blood stream, suggests strongly that two separate populations of lymphocytes contribute to the heterogeneous lymphocyte pool that is present in the node by the time of birth. It has been reported that both a short- and a long-lived population of lymphocytes is present in the circulating lymphocyte pool (Caffrey et al., '62) and that there is a difference in the immunological competence of these two populations (Gowans et al., '62). It seems likely that there is a variance in the functional capabilities of the two populations of lymphocytes in the developing lymph node. Several investigators have demonstrated the migration of chromosome-marked (Moore and Owen, '66) or radioactively-tagged hemopoietic cells (Murray and Murray, '64A) into lymphocytic tissues and organs. These findings do not preclude the in situ differentiation of lymphocytic cells from reticular cells, as in the lymph node and appendix (Ackerman, '66, '67B), or from undifferentiated epithelial cells as in the mammalian and avian thymus and in the avian bursa of Fabricius (Ackerman, '62; Ackerman and

Knouff, '64, '65; Auerbach, '60, '61). The local differentiation of lymphocytes in potential lymphocytic tissues is probably a short-term occurrence. It is of major importance only during certain periods of development of lymphocytic tissues in the embryo and is augmented later by a vascular infiltration of blood-borne lymphocytes.

Abbreviations

- S, Small-sized lymphocyte
- M, Medium-sized lymphocyte
- T, Cells considered transitional between reticular or mesenchymal cells and medium-sized lymphocytes
- E, Erythrocytic series
- F, Follicular artery
- R, Mesenchymal or reticular cell
- P, Post-capillary venule
- V, Lymphatic vessel
- D, Mesenchymal condensation
- Z, Cells considered transitional between undifferentiated epithelial cells and lymphoblasts in the thymus

PLATE I

Explanation of Figures

Figure 1. Transverse section through the abdominal aorta of a 17-day rabbit embryo showing the network of lymphatic vessels (V) and its relationship to the aorta. Note particularly the mesenchymal bridges between the lymphatic vessels. Toluidine blue staining following FSA fixation. X315

Figure 2. Area of developing lumbar lymph node from an 18-day rabbit embryo showing mesenchymal cell condensation (D) beside the enlarged lymphatic vessel (V). This area of condensation represents the early lymph node anlage. Stained with methylene blue following gluteraldehyde fixation and Araldite embedding. X640

Figure 3. Cross section through the abdominal aorta with its adjacent lumbar lymph node anlage. Note particularly the areas of mesenchymal condensation (D), i.e., the mesenchymal bridges or trabeculae. Nineteen-day rabbit embryo. Hematoxylin-eosin stain following acrolein fixation. X125

Figure 4. Plastic section from a 19-day embryo showing the presence of medium-sized lymphocytes (M) scattered randomly through the mesenchymal stroma of this developing, lumbar lymph node. Abdominal aorta is at the bottom of the picture. Basic fuchsin staining following gluteraldehyde fixation and Epon embedding. X640

PLATE I

PLATE II

Explanation of Figures

Figure 5. Section of an area of lumbar lymph node development from a 19-day rabbit embryo. Cells considered to be transitional (T) between the mesenchymal or reticular cell (R) and the medium-sized lymphocyte are seen. Three small-sized lymphocytes also may be seen (S). Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X1400

Figure 6. Lumbar lymph node from a 20-day rabbit embryo. Transitional forms (T) are evident in the midst of reticular (R) cells and medium-sized (M) lymphocytes. Note particularly the amount of basophilia, the chromatin pattern, and the thickness of the nuclear membrane in the transitional forms as compared to the reticular or mesenchymal cells. Comparison should also be made between transitional elements and the medium-sized lymphocytes (M). Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X1400

Figures 7 and 8. Lumbar lymph nodes from the lumbar region of a 21-day rabbit embryo. Note the trabecular arrangement of the stromal elements and the width of the developing sinuses. Transitional forms (T) are distinguishable. Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X800

PLATE II

PLATE III

Explanation of Figures

Figure 9. Twenty-one day rabbit embryo. Lumbar lymph node exhibiting transitional cells (T), and small (S) and medium-sized (M) lymphocytes. Note the trabecular arrangement of the stromal elements preparatory to the formation of cortical and medullary areas in the lymph node. Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X600

Figure 10. Twenty-one day rabbit embryo, lumbar lymph node. Note the small-sized lymphocyte in the developing sinus. Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X1400

Figures 11 and 12. Twenty-two day rabbit embryo, lumbar lymph nodes. Lymph node stroma is becoming more cellular as more transitional cells (T) and medium-sized lymphocytes (M) are seen in these trabeculae. Basic fuchsin staining following gluteraldehyde fixation and Araldite fixation. Figure 11, X1250. Figure 12, X600.

PLATE III

PLATE IV

Explanation of Figures

Figures 13 and 14. Lumbar lymph node from a 23-day rabbit embryo. Cells considered to be transitional (T) between mesenchymal cells and medium-sized lymphocytes are evident. Small and medium-sized lymphocytes are observed in this section (S, M). Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. Figure 13, X1200. Figure 14, X1400.

Figure 15. Twenty-four day rabbit embryo, lumbar lymph node. This picture shows several small lymphocytes (S) in the lumen of a small vein or capillary. The presence of lymphocytes in the blood vessels precedes the finding of lymphocytes in the walls of these vessels by one to two days. Basic fuchsin staining following Acrolein fixation and Epon embedding. X1400

Figure 16. Lumbar lymph node from a 25-day rabbit embryo, showing a small-sized lymphocyte (S) in the wall of a small artery. Several small lymphocytes are noted in the adjacent lymph node stroma. Basic fuchsin staining following Acrolein fixation and Araldite embedding. X1400

PLATE IV

PLATE V

Explanation of Figures

Figure 17. Lumbar lymph node from a six-week-old rabbit. Note the number of small lymphocytes (S) in the walls and directly adjacent to the post-capillary venule (P) as well as in the lumen of this vessel. Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X1400

Figure 18. Lumbar lymph node from a 27-day rabbit embryo showing a focus of erythrocytic cells in the medullary cord (E). Note that the series of erythrocytic cells are clustered and do not appear randomly throughout the lymph node stroma. Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X800

Figure 19. Spleen from a 25-day rabbit embryo showing the accumulation of small and medium-sized lymphocytes in the area adjacent to the follicular artery (S). Lymphocytes first appear in the developing spleen at 25 days of gestation. Basic fuchsin staining following gluteraldehyde fixation and Araldite embedding. X380

Figure 20. Thymus from a rabbit embryo of 19 days gestation showing cells considered to be transitional (Z) between undifferentiated epithelial cells and lymphoblasts. Several small and medium-sized lymphocytes also can be recognized. Basic fuchsin staining following gluteraldehyde fixation and Epon embedding. X800

33

28

PLATE V

APPENDIXES

APPENDIX A

Method for Embedding in Epon 812

Tissue blocks:	Fresh tissues minced to 1 mm. cubes.
Fixation:	2.5% Gluteraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 1% sucrose, 24 hours at 5° C.
Wash:	0.1 M cacodylate buffer (pH 7.2), 24 hours at 5° C.
Post fixation:	1% osmium tetroxide in 0.1 M Phosphate Buffer, pH 7.2, for 45 min. to 1 hour.
Dehydration:	Graded alcohols: 50% ethanol.... 30 min. 70% ethanol.... 30 min. 95% ethanol.... 30 min. 100% ethanol... 30 min. (2 changes, 15 min.) Propylene oxide 30 min. (2 changes, 15 min.)
Infiltration:	One part propylene oxide and one part Epon 812 resin. Mix by swirling and let stand for one hour. One part propylene oxide and 2 parts Epon 812 resin. Allow to infiltrate for 6 or more hours at 5° C.
Embedding:	Embed in Epon 812 resin. Polymerize in 60° C. oven for at least 48 hours.

APPENDIX B

Method for embedding in Araldite 502

Tissue blocks: Fresh tissues minced to 1 mm. cubes.

Fixation: 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 with 1% sucrose, 24 hours at 5° C.

Wash: 0.1 M. cacodylate buffer, pH 7.2, 24 hours at 5° C.

Post fixation: 1% osmium tetroxide in 0.1 M Phosphate Buffer pH 7.2 (Millonig's) for 45 min. to one hour.

Dehydration: Graded alcohols:

50% ethanol.... 30 min.
70% ethanol.... 30 min.
97% ethanol.... 30 min.
100% ethanol... 30 min.
(2 changes, 15 min.)
Propylene oxide 30 min.
(2 changes, 15 min.)

Infiltration: One part propylene oxide and one part Araldite 502 resin. Mix by swirling and let stand for four hours.

One part propylene oxide and two parts Araldite 502 resin. Mix and allow to infiltrate for 12 hours at 5° C.

Embedding: Embed in Araldite 502 resin.

Polymerize--60° C. oven for 48 hours.

APPENDIX C

Method for Preparation of Epon 812
and Araldite 502 ResinsEpon 812

Stock mixture No. 1: Epon 812..... 62 ml.
Dodecenyl succinic
anhydride (DDSA)..... 100 ml.

Stock mixture No. 2: Epon 812..... 100 ml.
Nadic methyl anhydride
(NMA)..... 89 ml.

Embedding mixture: Stock No. 1..... 5 ml.
Stock No. 2..... 5 ml.
Benzyldimethyl amine
(BDMA)..... 0.2 ml.

- Mix resins and anhydrides thoroughly.
- Mixtures are usable for several weeks if stored at 5° C.
- Allow bottles of propylene oxide and stock Nos. 1 and 2 to warm to room temperature before opening.

Araldite 502

Embedding mixture: Araldite 502..... 27 ml.
Dodecenyl succinic
anhydride (DDSA)..... 23 ml.
Dimethyl Amino methyl
phenol (DMP-30)..... 0.53 ml.

- Mix resin and anhydride thoroughly.
- Mixture will be stable for several weeks if stored at 5° C.
- Warm mixture to room temperature before use.

APPENDIX D

Method of Preparation of 0.1 M Cacodylate Buffer,
Glutaraldehyde Fixative, and Acrolein Fixative0.1 M cacodylate buffer:

Solution A: 4.28 grams sodium cacodylate in 100 ml.
distilled water.

Solution B: 2.4 ml. concentrated hydrochloric acid in
97.6 ml. distilled water.

To prepare: Mix the following:

Solution A..... 50 ml.
Solution B..... 2.4 ml.
Distilled water..... add to total 100 ml.

- Makes 0.1 M cacodylate buffer, pH 7.2-7.4.
- Check with pH meter.

Glutaraldehyde fixative:

Solution A: Stock solution of 25% glutaraldehyde.

Solution B: Cacodylate buffer, pH 7.2-7.4.

To prepare: Mix the following:

Solution A..... 10 ml.
Solution B..... 90 ml.
Sucrose..... 1 gm.

Acrolein fixative:

To prepare: Mix the following:

Acrolein, practical.. 10 ml.
Distilled water..... 90 ml.

- Use immediately.
- Use only freshly opened Acrolein stock
(less than two weeks old).

APPENDIX E

Method of Basic Fuchsin Stain for Plastic Sections

1. Cut sections at 1-2 micra and allow them to float out on 10% acetone.
2. Affix to glass slide by placing sections on drop of 40% acetone and heating for several minutes at 60° C.
3. Place slides in two changes of distilled water, 5 minutes each.
4. Place slides in filtered 2% basic Fuchsin in 50% acetone.
 - 30 min. in 60° C. oven for Epon 812
 - 20 min. in 60° C. oven for Araldite 502
5. Rinse well in 3 changes of distilled water.
6. Dehydrate through graded alcohols to absolute ethanol.
7. Clear in three changes of xylene.
8. Final xylene, 10 min.
9. Mount in synthetic mounting medium.

APPENDIX F

Method for Routine Double Embedding
with Parlodion and Paraffin

- Fixation: a) Formal-Sublimate-Acetic Acid, 24
 hrs., or
 b) 10% aqueous Acrolein, 1/2 to
 3 hrs.
- Wash: Distilled water.
- Dehydration: Graded Alcohols, 1-24 hrs., depending
 on size of tissue blocks.
- Infiltration I: 2%, 3%, and 4% parlodion, 1-3 days
 in each change depending on size of
 tissue.
- Clear: Three changes of benzene, 20 min. to
 1 hr. each, depending on size of
 tissue.
- Infiltration II: a) Equal parts benzene and paraffin
 for 1/2 hr., then
 b) three changes paraffin for 1/2 hr.
 each.
- Embed: Paraffin.

APPENDIX G

Rapid Method for Staining Plastic Embedded Tissues
for Light and Phase Microscopy

1. Dry slides thoroughly.
2. Cover sections with 1% aqueous methylene blue.
3. Add one drop of saturated solution of Alconox (Alconox, Inc., New York, New York).
4. Heat the slides by passing through a bunsen burner flame 12 times. DO NOT BOIL.
5. Rinse carefully with distilled water and let dry.
6. Mount in synthetic mounting media. Observe immediately.

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