LYMPHOPOIESIS AND LYMPHOCYTE RECIRCULATION IN THE SHEEP FETUS

By LEONARD D. PEARSON,* MAX W. SIMPSON-MORGAN,‡ AND BEDE MORRIS

(From the Department of Immunology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T.)

The immunologic capabilities of the fetal lamb have been documented in several studies (1–5), and it is certain that long before the lymphoid system has reached its full anatomical development, the fetus possesses an extensive repertoire of immune capabilities. This repertoire develops without exposure to antigen and in the absence of immunoglobulins.

The factors that are responsible for regulating lymphopoiesis are not known, but the thymus plays an important role in the generation of the normal complement of lymphocytes in the lamb, particularly throughout the last 100 days of pregnancy and in the first few weeks of postnatal life. Lymphopoiesis is severely reduced by thymectomy done between 60–70 days postconception (6).

Previous work established that it was possible to collect lymph from sheep fetuses continuously over periods of several days while the fetus remained within the uterus (7). The development of this technique provided a means whereby events occurring in the lymphoid system could be monitored continuously under physiological conditions. As little is known about the life history of lymphocytes in the fetal animal, we report here the results of experiments that were done to study aspects of lymphocyte recirculation and production in a situation where the lymphoid apparatus was still unsullied by contact with extrinsic antigen.

Materials and Methods

Animals. Merino ewes 3 yr and older were bred to Merino or to Border Leicester rams. The rams were fitted with marking harnesses and the ewes were observed daily to establish when they mated. Pregnancy was confirmed by failure of the ewe to mate again and by rectal-abdominal palpation (8). Pregnant ewes grazed outdoors and were given a supplement of lucerne hay. Before and after surgery they were kept in metabolism cages and fed lucerne chaff ad libitum and a supplement of grain oats.

Surgical Procedures. Operations were done on ewes carrying fetuses between 61 and 148 days gestation. Before surgery the ewes were deprived of food and water for 24–36 h. Thiopentone sodium ("Intraval" sodium, May and Baker, Ltd., West Foot Scray, Victoria.) was given intravenously to induce anesthesia, and halothane ("Fluothane," I.C.I. Australia Ltd., Sidney, New South Wales) and oxygen were administered in a closed circuit throughout the operation. All surgical procedures were done with strict asepsis.

Thoracic Duct Cannulation. The uterus was brought out through a ventral midline incision and placed in a polythene bag to keep it moist; it was then positioned on the abdomen of the ewe. The uterine wall and the amniotic sac were incised, taking care to avoid any major blood vessels or placental attachments. Hemorrhage was controlled by electrocautery. The amniotic fluid was usually removed and stored in a sterile flask at 37°C during the operation. For fetuses of 130–150 days of age, it was best to leave most of the fetal fluids and fetus itself within the uterus. The thoaracic duct was approached by removing the 7th or 8th rib on the right side and incising the costal perichondrium medial to the rib. When the ribs and lung were retracted the thoracic duct could be seen lying along the thoracic aorta. The parietal pleura over the duct was incised and bluntly dissected away and the duct ligated with a 4-0 silk thread as far cranially as possible. A second 4-0 silk thread was passed around the duct about 1 cm caudal to the first tie; the duct was then incised and cannulated. A 3-m length of polyvinyl tubing (inside diameter [ID] = 0.40–0.58 mm, OD = 0.80–0.96 mm, Dural Plastics Ltd., Dural New South Wales) was used for older fetuses and polyethylene tubing (ID = 0.28 mm, OD = 0.61 mm) was used for younger fetuses. The slightly beveled tip of the tube was passed posteriorly down the duct for a distance of 5–10 mm and then secured by the second tie. The tubing was brought out through the chest wall of the fetus and secured at this point to the skin. The chest wall of the fetus was then closed with silk sutures. The amniotic fluid was replaced, and the placental membranes were closed with a series of purse-string ligatures. It was important to insure that there was no leaks in the fetal membranes, for fetuses less than 130 days postconception were aborted if fluid leaked from the amniotic sac. The tubing was brought to the outside by passing it through the paralumbar fossa of the ewe and out through the skin. Loose coils were left within the uterus and the abdominal cavity of the ewe to allow for any movements of the fetus or the uterus. The uterine and abdominal incisions in the ewe were closed with silk sutures. From the outset, lymph was collected quantitatively in a collection bottle which was attached to the side of the metabolism cage. The experimental preparation is shown diagrammatically in Fig. 1.

Thymectomy. Thymectomies were performed as described by Cole and Morris (6). The cranial part of the thymus of the lamb extends along the entire length of the neck in close association with the trachea; the thoracic part lies within the mediastinum anterior to the heart. The entire organ was removed.

Cannulation of the Jugular Vein. A jugular vein in each fetus was cannulated with polyvinyl tubing. To prevent clotting between withdrawal of blood samples the lumen of the cannula was filled with heparinized saline (200 U/ml).

Counting Thoracic Duct Lymph Cells. Lymph was collected in tared bottles containing approximately 10 mg of a mixture of 100,000 U of freeze-dried heparin ("Pularin", Evans Medical Ltd., Liverpool, England), 1,000,000 U of crystalline penicillin G, and 500,000 U of streptomycin sulphate. Cells were diluted 1:100 or 1:200 and counted with a Coulter counter (Model FN, Coulter Electronics Inc., Hialeah, Fla.). Smears of cells were stained with Leishman's stain.

Labeling Thoracic Duct Lymph Cells in Vitro. Cells for labeling were taken from collections of lymph made over periods of 24–48 h at varying times after cannulation. The samples were centrifuged at 400 × g for 5 min and the cells resuspended in autologous lymph. The concentration of cells varied from 9.6 × 10^7 to 2.4 × 10^8 per ml. [5-^{3}H]uridine (27 Ci/mmol) or [6-^{3}H]cytidine (7.5 Ci/mmol, Radiochemical Centre, Amersham) was added to the cell suspension to give a concentration of 20–30 μCi/ml, and then the cells were incubated for 1 h at 37°C in sterile 4-ml flat-bottomed glass vials. The contents of the vial were gently shaken at 10-min intervals. After the incubation period the cells were washed five times with 10-ml vol of autologous lymph. They were finally washed in Hanks' balanced salt solution at pH 7.4 and then counted. Almost all lymphocytes were labeled by these procedures, although there was considerable variation between cells in the amount of isotope taken up. Cytidine proved to be a better label for sheep lymphocytes than uridine.

Some samples of thoracic duct lymph cells were also labeled with iodinated deoxyuridine. Cells were suspended at a concentration of 6.0 × 10^7 cells/ml in 30 ml of L-15 medium (Leibovitz medium, Grand Island Biological Co., Grand Island, N. Y.) containing 15% fetal calf serum and 1 μCi/ml of ^{125}I-5-iodo-2-deoxyuridine (72 Ci/g, Radiochemical Centre, Amersham) and incubated for 8 h at 37°C. The labeled cells were then washed seven times in 10-ml vol of autologous lymph, counted, resuspended in 10 ml of L-15 medium without added serum, and infused back into the
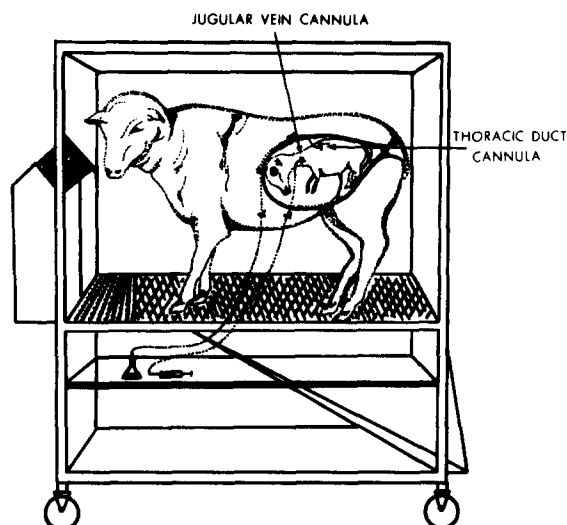
FIG. 1. Diagram of the experimental model. The fetus in utero carries indwelling plastic catheters in the thoracic duct and in the jugular vein from which blood and lymph samples can be collected over periods of weeks.

donor fetus via the jugular cannula over a 15-min period using a slow injection apparatus (Sage Instruments, Cambridge, Mass.). Between 85–95% of the cells were not stained by trypan blue when tested immediately before they were infused.

Autoradiography. Lymphocytes labeled with [³H]uridine or [³H]cytidine were smeared onto glass slides, dried in air, and then fixed in methanol for 5 min. The slides were dipped in Ilford K5 emulsion (Ilford, Ltd, Ilford, Essex, England) or coated with Kodak AR 10 stripping film (Kodak Ltd, London, England) and exposed for periods of 1–4 wk. After development in Kodak D19 developer, the slides were washed in water for 20 min and then stained with 0.1% azure A in acetate buffer, pH 5.2.

Tissues were fixed in formol-saline, embedded in paraffin wax, and cut at a thickness of 5 μm. The sections were treated with xylol to remove the wax, coated with gelatin, and overlaid with Kodak AR 10 stripping film. They were stored for 4–8 wk in light-tight boxes and then developed with Kodak D19 developer. Sections were stained with 0.1% azure A.

Scintillation Counting. The cell content of lymph from fetuses that received lymphocytes labeled with [³H]uridine or [³H]cytidine was measured with a Coulter counter. Samples were centrifuged at 400 × g for 10 min in 15-ml conical tubes and cell pellets transferred quantitatively with washes of preinfusion lymph onto 3 × 8-cm strips of Whatman chromatography paper. The dried paper strips bearing the cells were then oxidized in a Model 305 oxidizer (Packard Instrument Co., Inc., Downers Grove, Ill.). A liquid scintillation spectrometer (Model 3320, Packard Instrument Co., Inc.) was used to measure radioactivity in the samples prepared by the oxidizer.

Cells in lymph from fetuses that received lymphocytes labeled with ¹²⁵I-deoxyuridine were counted with the Coulter counter and then placed in 15-ml disposable plastic tubes. Gamma emissions were counted in a scintillation spectrometer (Model 578, Packard Instrument Co., Inc.) that was calibrated for ¹²⁵I.

Results

The Output of Lymphocytes in the Thoracic Duct Lymph of Normal Sheep Fetuses. Lymph was collected from 17 normal fetal lambs whose ages ranged from 75–148 days postconception (150 days is the normal gestation period of sheep). The number of cells in the lymph was measured for cumulative collection periods at 6, 12, 24, 36, and 48 h after cannulation and then daily. Cannulas
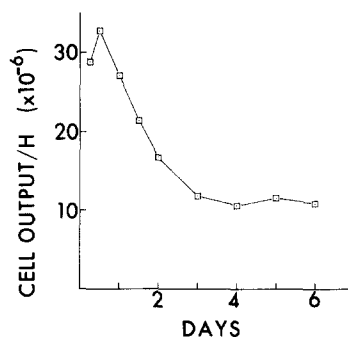
FIG. 2. The cell output in the thoracic duct lymph of a fetus cannulated at 96 days gestation.

TABLE I

The Concentration of Cells, the Rate of Flow, and the Output of Cells in the Thoracic Duct Lymph of Normal Sheep Fetuses 70–150 Days of Gestational Age as Predicted by Regression Equations Relating Log $_e$ Cell Concentration and Log $_e$ Lymph Flow to the Fetal Age*

Days	Cells/ml × 10^{-6} ‡	ml/h‡	Cells/h × 10^{-6} ‡
70	2.0 (1.7–2.4)	0.7 (0.2–2.5)	1.5 (0.4–6.0)
80	2.4 (2.0–2.9)	1.1 (0.3–3.7)	2.7 (0.7–10.6)
90	2.9 (2.5–3.4)	1.7 (0.5–5.4)	5.1 (1.4–18.7)
100	3.5 (3.0–4.1)	2.6 (0.9–8.1)	9.3 (2.6–33.7)
110	4.3 (3.6–5.0)	4.1 (1.3–12.3)	17.3 (4.9–61.5)
120	5.1 (4.4–6.0)	6.2 (2.0–18.9)	32.0 (9.0–113.9)
130	6.2 (5.3–7.3)	9.5 (3.1–29.5)	59.3 (16.4–214.4)
140	7.5 (6.4–8.8)	14.6 (4.6–46.4)	109.7 (29.4–409.4)
150	9.0 (7.6–10.7)	22.4 (6.8–74.1)	203.0 (52.0–792.9)

* The calculated regression equations were $\log_e$ cells/h = 0.062 × −3.920 and $\log_e$ ml/h = 0.043 × −3.302, where X = days of gestation (standard errors for B were 0.006 and 0.006, respectively.

‡ 95% confidence interval, in parenthesis.

were difficult to keep patent in the youngest fetuses; two fetuses flowed for 6 h, three for 24 h, seven for up to 5 days, and five for periods from 7 days to 36 days. Some lambs were born with their cannulas intact and lymphatic-venous shunts were established in these after birth to return the lymph to the blood stream.

The output of cells decreased rapidly over the first 4 days of drainage to reach a level around 25–30% of the first 24-h output (Fig 2). After this time the daily cell output remained relatively constant for periods of up to several weeks. The cell outputs and the rates of lymph flow during the first 24-h periods of collection were plotted against the ages of the fetal lambs and linear regression equations were fitted to the $\log_e$ transformed data. Both the output of cells and the rate of lymph flow increased exponentially with the age of the fetus. The predicted values for the concentration of cells in the thoracic duct lymph, the rate of lymph flow, and the output of cells per hour for fetuses from 70–150 days postconception were calculated from the regressions and are given in Table I. The concentration of cells in the lymph increased about fivefold between 70 and 150 days of gestation, while the rates of lymph flow increased approximately 20-fold during
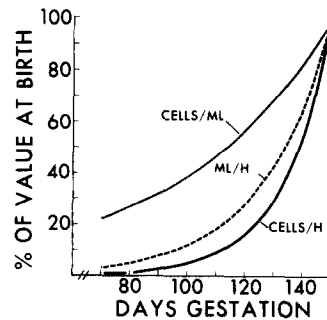
Fig. 3. Cell concentration, flow rate, and cell output of thoracic duct lymph from normal sheep fetuses expressed as a percentage of the expected values at birth. The curves are drawn from the calculated regression equations given in Table I.

the same. There was, in consequence, a very large increase in the output of cells in the thoracic duct lymph over the last 70 days of gestation. These changes are shown in Fig. 3.

The size of the circulating pool of readily mobilized lymphocytes was calculated from the total number of cells collected in the thoracic duct lymph during the first 4 days of cannulation. During this time the lymphocyte output had fallen to a relatively steady plateau. Fetuses of 95–100 days of age had a mean of $5.5 \pm 1.5 \times 10^8$ (SE) cells in this pool; for fetuses of 130–135 days there were $5.7 \pm 1.2 \times 10^9$ cells, while in fetuses of 145–150 days, the pool size was $1.2 \pm 0.3 \times 10^{10}$ cells.

The Effect of Chronic Thoracic Duct Drainage on the Output of Lymphocytes. After the first few days of drainage the daily output of cells in the lymph was maintained at a steady level over periods of weeks. In fact, in those fetuses drained for the longest periods of time the cell output tended to increase gradually. After the first 1–2 wk the output of cells in the lymph was taken to reflect the rate at which lymphocytes were being produced and added to the pool of cells that was mobilized into the thoracic duct by continued drainage. The rate at which lymphocytes entered the thoracic duct lymph was $3.2 \pm 1.9 \times 10^6$ cells/h in fetuses near 100 days of age and $3.4 \pm 0.9 \times 10^7$ cells/h in fetuses near 130 days. In one fetus that was drained for 36 days from 96 days to 132 days postconception, the cell output in the lymph increased from 1.1×10^7/h on day 100 to 2.4×10^7 on day 130.

The Histology of Lymphoid Tissues After Chronic Thoracic Duct Drainage. The lymphoid tissues of fetuses that had been drained for varying periods of time showed only minor changes in their content of lymphoid cells. The tissues of the fetus that underwent continuous drainage for 36 days were compared at the end of this time with a normal fetus of the same age (Fig. 4). During the period of lymph drainage, 1.5×10^{10} cells weighing approximately 3.75 g were collected. In spite of this loss, lymphocytes were plentiful in the various lymphoid tissues. The density of lymphoid cells in the thymic cortex and the medulla of the chronically drained lamb was not reduced significantly (Fig. 4 A and B). The thymuses of other fetuses drained for periods of time varying from 1–5 wk showed little change from normal.
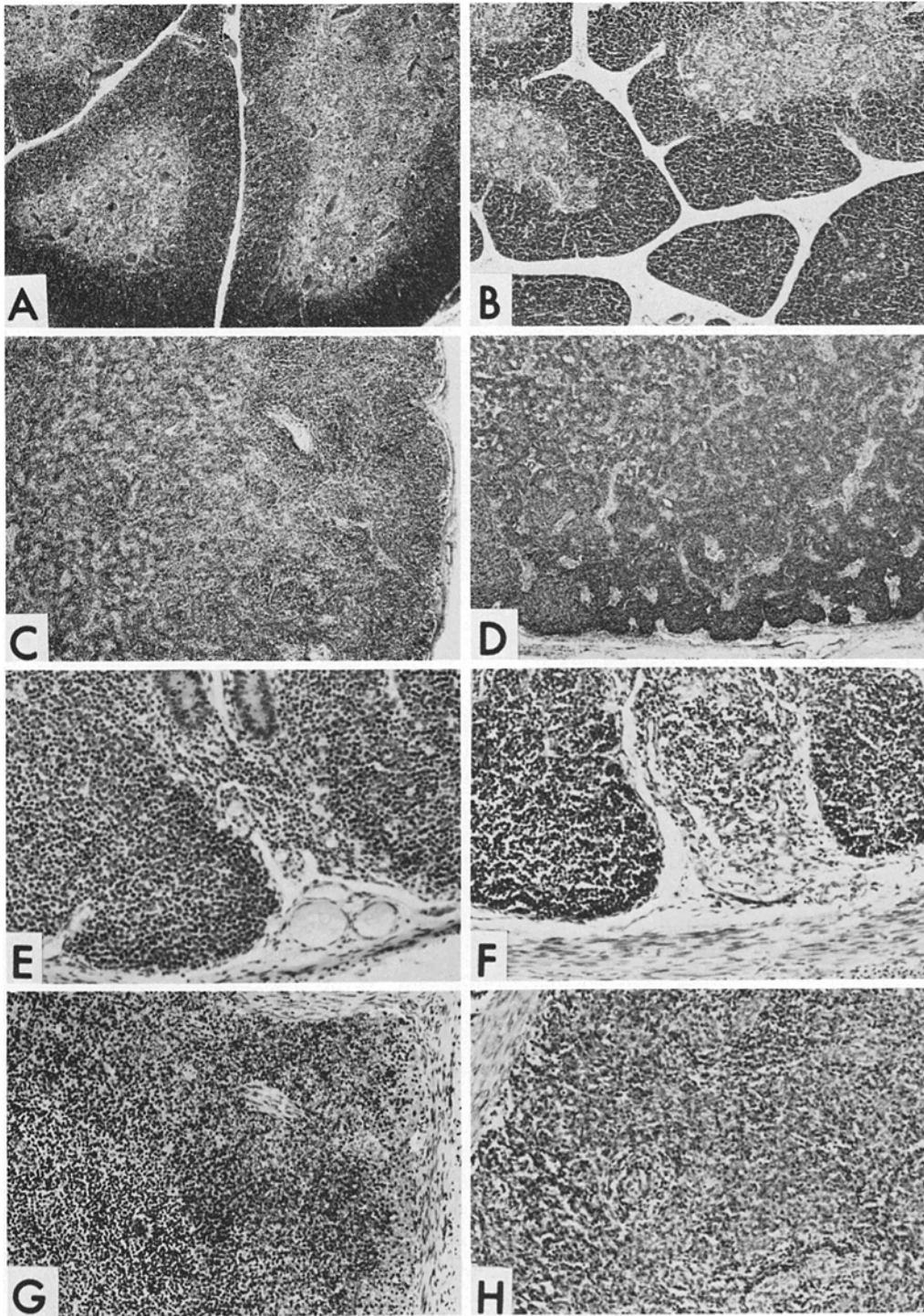
The lymph nodes of the fetus drained for 36 days were differentiated into cortical and medullary areas; there was no obvious reduction in the number of lymphocytes in the cortico-medullary region compared to the normal lamb of the same age (Fig. 4 C and D). Peyer's patches had developed in both the drained fetus and the normal fetus (Fig. 4 E and F). There appeared to be fewer lymphocytes in the interfollicular areas of the Peyer's patches and the periarteriolar areas of the spleen in the drained fetus, although this difference was not great (Fig. 4 G and H).

The cell population of the thoracic duct lymph of the fetal lamb comprised more than 98% small lymphocytes. In fetuses subjected to thoracic duct drainage there was no evidence that increasing numbers of large cells were being added to the lymph as the recirculating pool became depleted of cells. The fetus that was drained continuously for 36 days had only 4% large cells in the thoracic duct lymph at the end of this time.

The Recirculation of Lymphocytes in the Sheep Fetus.   All of the lymphocytes collected over the first 24 h after thoracic duct cannulation in a 96 day fetus were incubated with [³H]uridine to label them (Fig. 5 A). These labeled cells were then infused back into the donor intravenously and their reappearance was subsequently followed in the thoracic duct lymph. The labeled cells were detected in the lymph in the first hour, and they reached maximum numbers approximately 10–18 h after the infusion began (Fig. 5 B and 6).

Two fetuses were cannulated at 145 and 148 days postconception; lymphocytes were collected from these during the first 24 h after cannulation, labeled separately with ¹²⁵I-iododeoxyuridine and with [³H]cytidine, and infused intravenously back into the donor fetus. The results of these experiments were similar; the labeled cells first appeared in the thoracic duct lymph within 60 min after the infusion began. In the fetus infused with [³H]cytidine-labeled cells, 16% of the cells in the lymph were labeled cells by 18 h after the infusion ceased.

The rates at which the labeled cells returned to the thoracic duct lymph were compared by plotting the cumulative recoveries of radioactivity against time (Fig. 7). The slopes of the three curves were very similar over the first few hours and during this time about 1.7% of the labeled cells infused into the bloodstream reappeared in the lymph each hour. The total cumulative recoveries of labeled cells in the older fetuses were significantly less than in the younger fetus, but

FIG. 4.   The histological appearance of the lymphoid tissues of a sheep fetus at 132 days gestation subjected to continuous thoracic duct lymph drainage for the previous 36 days. The histology of lymphoid tissues from a normal fetus of 132 days gestation are shown for comparison. (A) Thymus from the normal fetus (Hemotoxylin and eosin [H & E] × 20). (B) Thymus from the drained fetus showing a similar structure and cellularity to (A) (H & E × 20). (C) Mesenteric node from the normal fetus with primary follicles in the cortex. The definition between cortex and medulla is indistinct (H & E × 20). (D) Prescapular node from the drained fetus similar in appearance to (C) (H & E × 20). (E) Peyer's patch from the ileum of the normal fetus (H & E × 80). (F) Peyer's patch from the drained fetus. There are slightly fewer lymphocytes in the interfollicular areas than in (E) (H & E × 80). (G) Spleen from the normal fetus. Lymphocytes surround the periarteriolar sheaths (H & E × 80). (H) Spleen from the drained fetus. The periarteriolar sheaths were judged to have fewer lymphocytes than normal (H & E × 80).
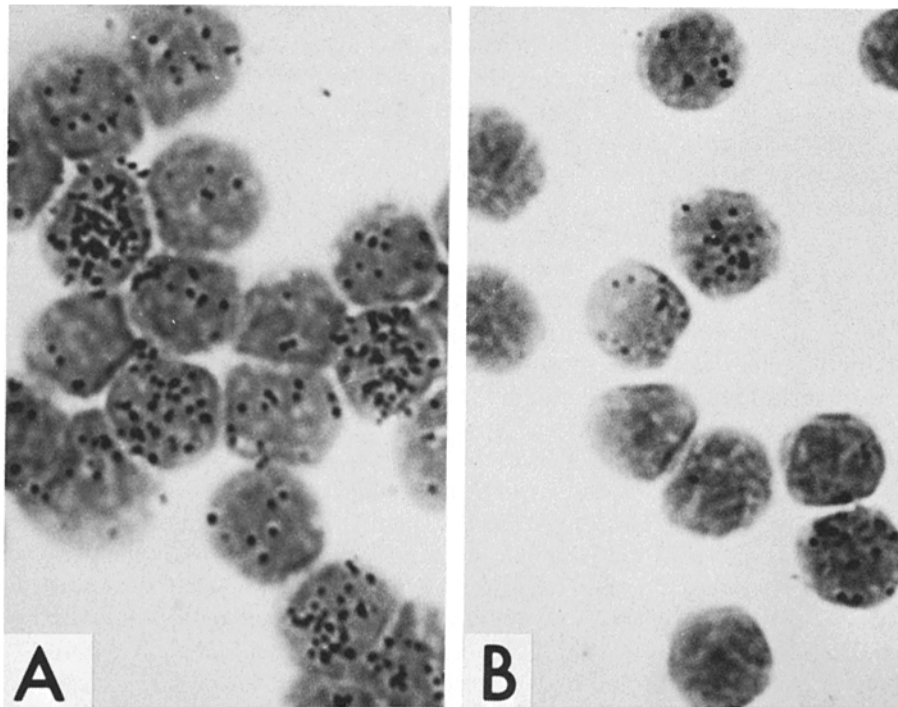
FIG. 5. (A) Autoradiography of thoracic duct lymphocytes from a sheep fetus collected during the first 24 h after cannulation. The cells were labeled in vitro with [3H]cytidine and injected back into the donor fetus and their subsequent appearance followed in the thoracic duct lymph (azure A × 1,400). (B) Autoradiograph of cells from thoracic duct lymph of a fetus 18 h after intravenous infusion of cells shown in (A). At this time 16% of the cells in the lymph were labeled (azure A × 1,400).
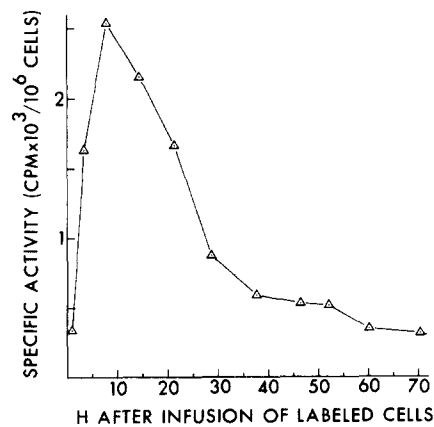

FIG. 6. Recirculation of lymphocytes in a normal sheep fetus of 97 days gestation. Cells collected during the first 24 hr of thoracic duct drainage were labeled with [3H]uridine before they were infused intravenously back into the fetus.
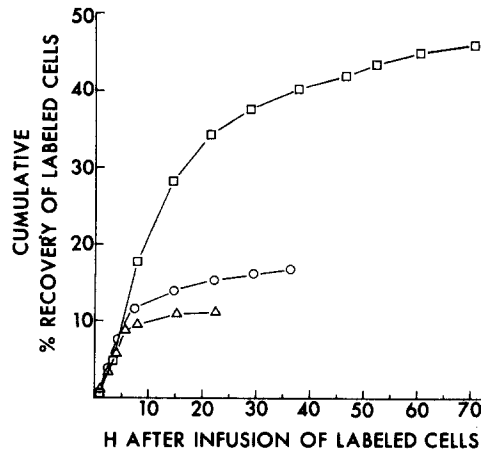
FIG. 7. Cumulative recoveries of labeled lymphocytes in the thoracic duct lymph of normal sheep fetuses. The results of three experiments are shown: (□) Fetus of 97 days gestation infused with cells labeled with [³H]uridine. (○) Fetus of 145 days gestation infused with cells labeled with [³H]cytidine. (△) Fetus of 148 days gestation infused with cells labeled with ¹²⁵I-iododeoxyuridine.

these experiments were terminated when the lambs were born 24–36 h after the cell infusion began.

Tissues from two lambs infused with labeled lymphocytes were fixed in formol-saline and sections examined by autoradiography. Labeled lymphocytes were found in all the various lymph nodes and the gut; very few were found in the thymus or in the spleen. There were more labeled cells in the mesenteric lymph nodes and in the Peyer's patches than in the other lymphoid tissues. In the mesenteric nodes labeled cells were concentrated in the primary follicles of the cortex (Fig. 8 A), although some were also scattered throughout the cortex. In the Peyer's patches the labeled cells were localized in the interfollicular areas; there were none in the follicles (Fig. 8 B).

The Output of Lymphocytes in the Thoracic Duct Lymph of Thymectomized Fetuses. Thoracic duct cannulations were done on five thymectomized fetal lambs between 124–126 days postconception. These fetuses had been thymectomized at 61–83 days (Table II). The cell output in the thoracic duct lymph of each thymectomized fetus was significantly less than normal. As in normal fetuses, cell outputs were highest during the first 24 h after cannulation and fell rapidly to a relatively steady level after 3–4 days of drainage (Fig. 9). The cell concentrations in the thoracic duct lymph, the rates of lymph flow, and the cell outputs during the first 24 h of drainage were compared with the values for normal fetuses cannulated at 125 days postconception (cf. Table I). The outputs of lymphocytes in the lymph of four fetuses were 14–23% of the normal level. The cell output in the fetus that was thymectomized at 83 days postconception was 42% of normal (Table II).

The Effect of Chronic Thoracic Duct Drainage on the Output of Lymphocytes in Thymectomized Fetuses. Lymph was drained for 1.5, 3, 15, 16, and 24 days from the five thymectomized fetuses. The cell outputs over 2 wk of continuous drainage were compared to the cell outputs of normal fetuses of equivalent age.
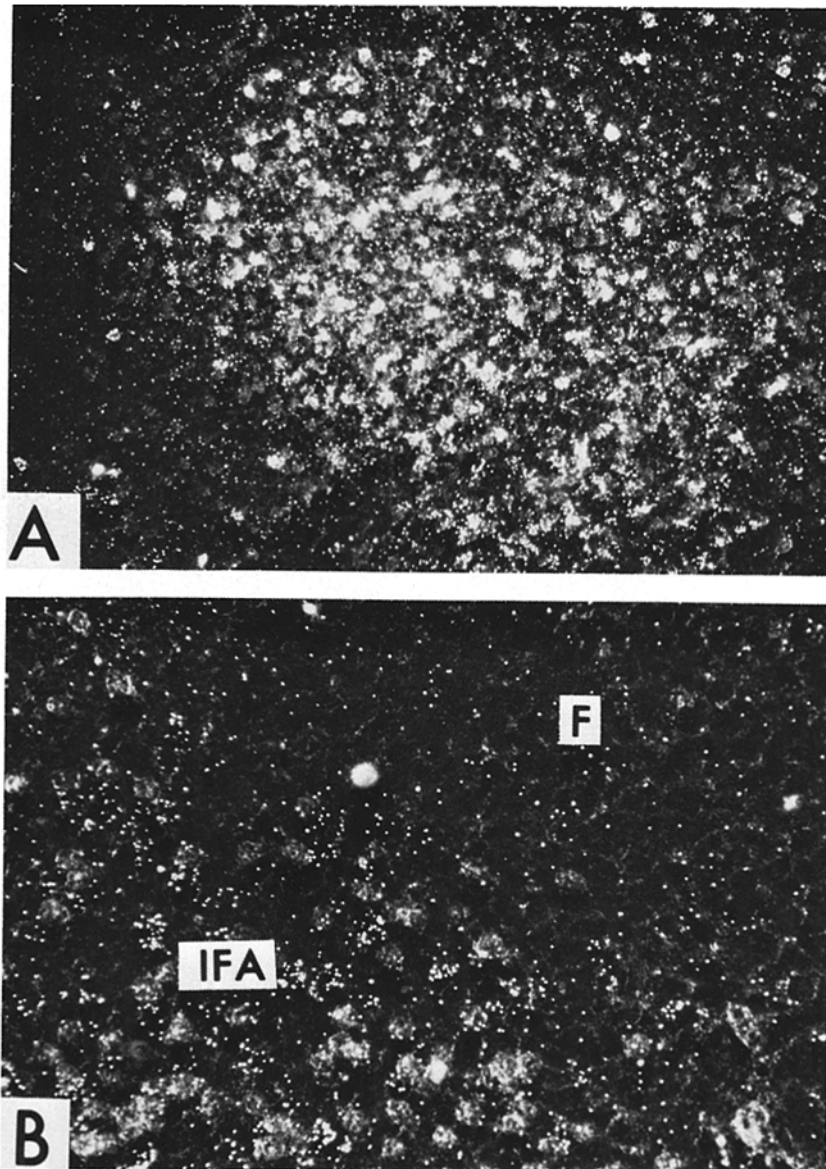
FIG. 8.   The accumulation of [³H]cytidine-labeled lymphocytes in a primary follicle of the mesenteric node of a fetal lamb 145 days gestation, 18 h after infusion of the labeled cells intravenously (dark field × 400). (B) [³H]cytidine-labeled lymphocytes in the interfollicular area (IFA) of a Peyer's patch in the ileum of the same fetal lamb as in (A) 18 h after infusion of the labeled cells intravenously. The follicle (F) of the Peyer's patch is devoid of labeled cells (dark field × 600).

The thymectomized fetuses had cell outputs which were around 25% or normal during the first 24 h after cannulation. After 14 days of drainage, the cell output in the fetuses near to term had fallen to a steady level of 2–4 × 10⁶ cells/h; this was about 10% of the output of normal fetuses.

TABLE II

The Concentration of Cells, the Rate of Flow, and the Output of Cells in the Thoracic Duct Lymph of Thymectomized Fetal Sheep

Age at Thy-X	Age at cannula-tion	Cells/ml × 10⁻⁶	ml/h	Cells/h × 10⁻⁶
Not Thy-X*	125	5.6	7.7	43.6
61	125	1.7	5.7	9.8
67	124	1.3	4.6	6.0
70	126	0.6	15.7	9.5
80	125	1.1	7.2	8.0
83	125	1.1	16.5	18.2

* The mean values for normal fetuses of 125 days gestation are given for comparison.
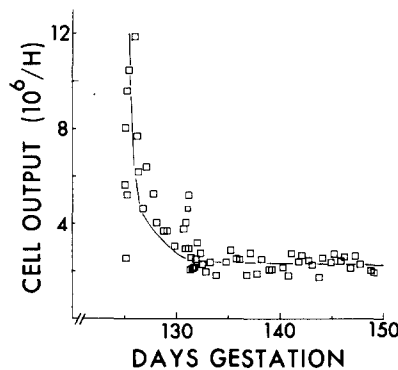

FIG. 9. Cell output in thoracic duct lymph of a fetus thymectomized at 61 days and subsequently cannulated at 125 days gestation.

The Effect of Thymectomy and Chronic Drainage on the Histology of Lymphoid Tissues. The histological appearance of the thymuses at the time 'they were removed is shown in Fig. 10 A. All the structures of the mature gland were present in the fetal thymus from 61 days onward, although in younger fetuses the development of the cortex was not complete and the gland lacked its full content of lymphocytes. The weight of the thymus in 61-day fetuses was 0.5 g and was 1.7 g in the 83-day fetus.

The lymphoid tissues taken from thymectomized lambs that had undergone chronic thoracic duct lymph drainage 42–64 days after thymectomy had reduced numbers of lymphocytes (Fig. 10 B–F). The follicular and interfollicular areas of the Peyer's patches were depleted of lymphocytes (Fig. 10 B), and there were fewer lymphocytes than normal in the cortical and paracortical areas of lymph nodes (Fig. 10 C and E). The number of lymphocytes in the periarteriolar areas of the spleen was also reduced (Fig. 10 D and F). There were, however, no obvious differences in the histological features of lymphoid tissue from fetuses drained for varying periods of time from 3–17 days (compare Fig. 10 C and D with E and F), and this suggested that most of the alterations in the tissues were
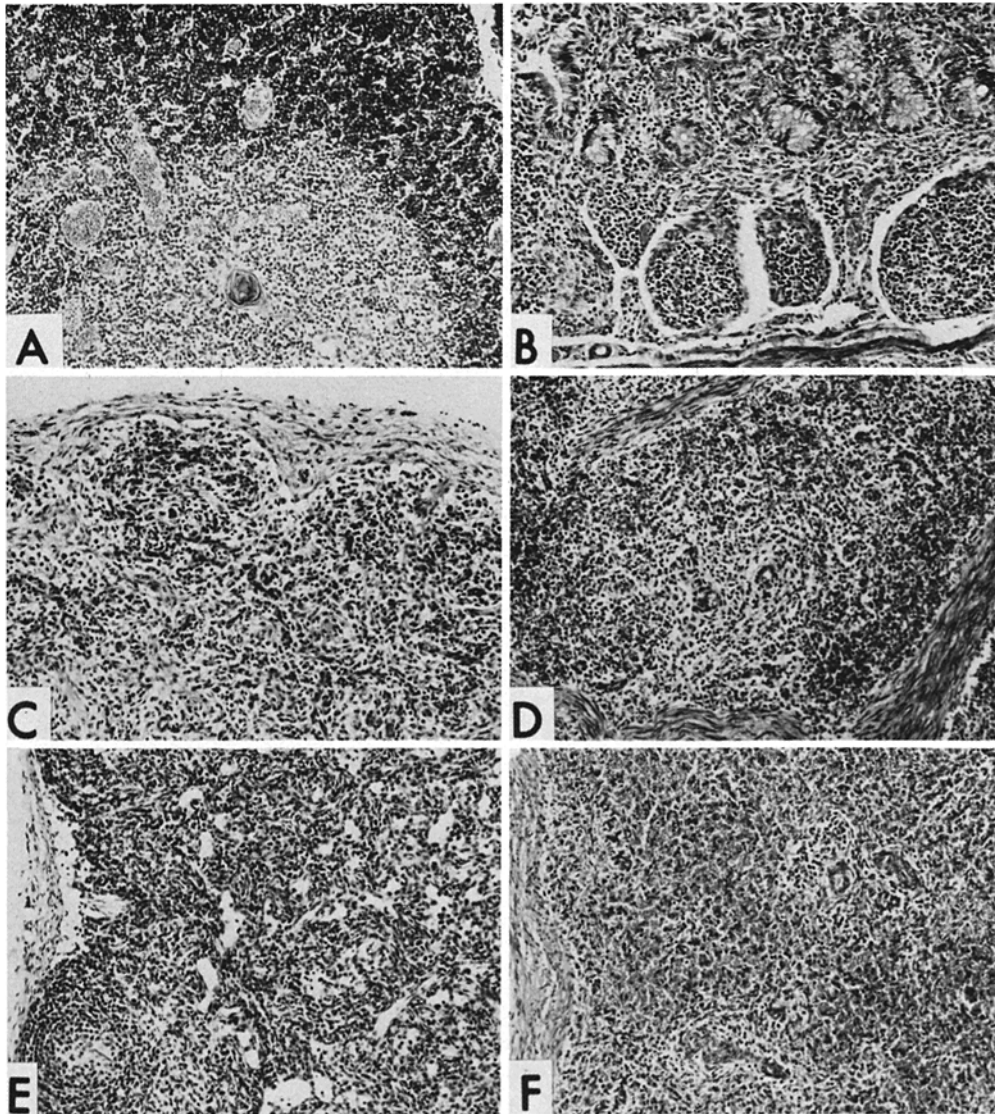
FIG. 10. The histological appearance of the lymphoid tissues of thymectomized fetuses subjected to thoracic duct lymph drainage. (A) Thymus from a fetus at 70 days gestation showing cortex and medulla with Hassal's corpuscles (H & E × 80). (B) Peyer's patches from the ileum of a thymectomized fetus of 142 days gestation. Thymectomy was done at 70 days, the thoracic duct cannulated at 126 days, and lymph drained for 16 days. There are fewer lymphocytes in the follicles and interfollicular areas than normal (H & E × 80). (C) The popliteal lymph node of a thymectomized fetus at 128 days gestation. Thymectomy was done at 80 days, the thoracic duct cannulated at 125 days, and lymph drained for 3 days. Lymphocytes are depleted from the cortex and paracotrex (H & E × 80). (D) The spleen from the same fetus as (C). Lymphocytes are depleted from the periarteriolar sheaths (H & E × 80). (E) The prescapular lymph node of a thymectomized fetus of 141 days gestation. Thymectomy was done at 67 days, the thoracic duct cannulated at 124 days, and lymph drained for 17 days. Lymphocytes are depleted from the cortex and medulla, but the number of these cells is similar to that in the thymectomized fetus drained for 3 days (C) (H & E × 80). (F) The spleen from the same fetus as (E). The number of lymphocytes present is similar to that in the thymectomized fetus drained for 3 days (D) (H & E × 80).
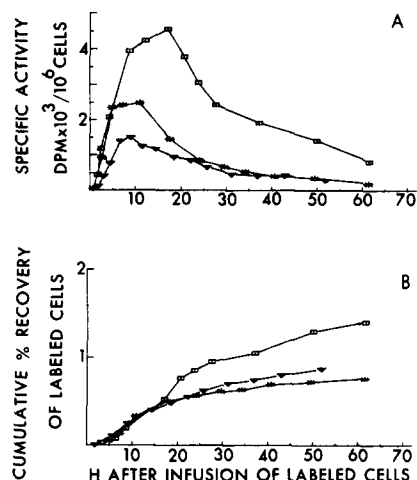
178

FIG. 11. (A) Recirculation of [³H]uridine-labeled lymphocytes from blood to lymph in thymectomized fetuses. (□) Thymectomized at 70 days, thoracic duct cannulated at 126 days and labeled cells infused at 129 days. (*) Thymectomized at 67 days, thoracic duct cannulated at 124 days and labeled cells infused at 130 days. (∇) Thymectomized at 61 days, thoracic duct cannulated at 125 days and labeled cells infused at 131 days. (B) Cumulative recoveries of [³H]uridine-labeled cells in the thoracic duct lymph of the thymectomized fetuses infused with labeled cells intravenously.

due to the previous removal of the thymus rather than to the effects of thoracic duct drainage.

The Recirculation of Lymphocytes in the Thymectomized Sheep Fetus. Lymphocytes from the thoracic duct lymph of thymectomized fetuses were labeled in vitro with [³H]uridine. When these labeled cells were injected intravenously back into the donor fetus they reappeared in the lymph in the first hour (Fig. 11 A). The general shape of the curves relating the specific activity of the cell population in the lymph with time was similar to that in normal fetuses, and any differences in these curves in thymectomized fetuses reflected differences in the total radioactivity and in the number of cells infused intravenously. The maximum specific activity of the cells in the lymph of thymectomized fetuses occurred 18–22 h after the labeled cells were infused, and this was around the same time as in normal fetuses. It was calculated that 0.03% of the labeled cells reappeared in the lymph each hour during the period 5–15 h after the cells were infused. Less than 2% of the total label infused into the thymectomized fetuses was recovered in the thoracic duct lymph over the 60-h period after injection (Fig. 11 B); this was significantly less than from normal fetuses.

Discussion

Drainage of the thoracic duct lymph from fetal lambs in utero even for periods as long as 5 wk had no apparent detrimental effects on their growth or development. Fetuses with chronic lymphatic fistulas had normal birth weights, and those that were born with the lymphatic cannula intact were vigorous and healthy. When the lymphatic fistula was closed after birth, the number of lymphocytes in the blood returned rapidly to normal values. No nutrients,

electrolytes, or fluids were given directly to the fetuses at any stage, yet lymphocyte production and lymph flow were sustained at a steady level over long periods of time. In the fetus drained for 36 days, more than 12 liters of lymph (about 15 times the average body weight over the drainage period) and about 350 g of plasma protein were collected without compromising its growth or development. The apparent indifference of the fetal lamb to the removal of large quantities of fluids, electrolytes, and protein by way of a thoracic duct fistula is in contrast to the dire effects of this procedure in animals after birth. Smeaton et al. (7) noted that in near term fetal lambs thoracic duct drainage produced a fall in the protein content of the lymph after 1–2 days. It appears that with continued drainage, the protein content of the lymph stabilizes at a lower level and is maintained at this steady state for long periods of time.

In small animals such as rats and mice various procedures are used to enhance the rate of lymph flow and to restrict or stimulate the movements of the cannulated animals. This must result in a variety of stresses to the lymphoid apparatus which may compromise its function and confound the effects of lymphocyte depletion. Even with continuous infusions of fluids and electrolytes, or with the return of the lymph, losses in body weight in these animals are usually quite severe if drainage is continued over periods of weeks. It seems that the fetal lamb is protected from these untoward effects within the uterus, and it is the ewe that bears the brunt of the losses sustained by the fetus through the thoracic duct fistula. Most ewes carrying fetuses with cannulated thoracic ducts ate and drank voraciously, but many still lost weight.

Lymphocyte Output in the Thoracic Duct Lymph. Lymphocytes can be detected first in the thymus of the fetal lamb at around 40 days postconception and are present in the spleen and lymph nodes at 50–60 days (2, 9). The youngest fetus from which we collected lymph was 75 days postconception, and at this age approximately 1×10^6 cells were passing through the thoracic duct each hour. It is certain that lymphocytes would be present in the lymph of fetuses younger than 75 days, and in all probability at least some would be present at around 30–40 days when these cells first appear in the blood stream. The cell output in the thoracic duct lymph increased exponentially throughout gestation in a similar way to the growth of the lymphoid organs, indicating a close relationship between the size of the recirculating pool of lymphocytes and the total mass of lymphoid tissue.

When guinea pigs, calves, mice, and rats are subjected to chronic thoracic duct drainage, the output of lymphocytes in the thoracic duct lymph has been shown to decrease rapidly during the first few days and after this to decline at a much slower rate (10–14). After the first 4 days of thoracic duct drainage the output of cells in the lymph of the fetal lamb was maintained at a steady state over periods of weeks; the output of cells during this time was taken as the rate at which new lymphocytes were being added to the recirculating pool of cells. These cells could have been derived from a pool of lymphocytes that were drawn very slowly into the circulation by the continuous drainage of lymph or they could have been newly formed cells. Gowans and Knight (15) examined the origin of lymphocytes in rats subjected to thoracic duct drainage for 4 days by giving them [3H]thymidine intravenously on the 5th day. They found that more

than 87% of the small lymphocytes in the lymph were still unlabeled in collections made on the 5th and 6th day and concluded from this result that those lymphocytes appearing in the thoracic duct lymph after 4 days of drainage were not newly formed cells but cells released slowly into the recirculating pool from the tissues. While there is a pool of lymphocytes in the tissues from which cells are mobilized slowly into the circulation, the fact that the cell output in the lymph remained stable or gradually increased over periods of drainage of 3–5 wk seemed most likely to be due to the addition of newly-formed lymphocytes to the blood and lymph. Because the spleen and lymph nodes increased significantly in size and in their content of lymphocytes during the period of drainage, it was certain that newly-formed cells were being added to the fixed component of the lymphoid tissues as well as to the recirculating pool of cells.

The great reduction in the numbers of cells in the recirculating pool in thymectomized fetuses and the much slower rate of addition of new lymphocytes to this pool after thymectomy indicated that in the fetal lamb the thymus was responsible for the production of the majority of the cells that are normally undergoing recirculation from the blood to the lymph. However, thymectomy did not entirely prevent the expansion of the recirculating lymphocyte pool nor did it stop the accumulation of cells in lymphoid tissues; thus a significant number of lymphocytes were being produced in extrathymic tissues in the fetus in the absence of any antigenic stimulation.

Estimates have been made of the number of cells leaving the thymus in several species of animals by measuring the content of lymphocytes in the thymic arterial and venous blood (16–19). In addition, indirect measurements have been made of the extent of lymphocyte migration from the thymus by way of the lymph stream (20). Even though measurement of thymic blood flow and arteriovenous differences in cell content would be subject to considerable error, these studies have all indicated that the rate of lymphocyte migration from the thymus in various species is of the order of $0.5–1.0 \times 10^6$ cells/mg of thymus per day (21). These experiments provided no indication of how many of the cells that left the thymus by way of the blood or the lymph actually entered the recirculating pool, although it has been shown that at least some cells produced within the thymus do so (22–24). However, in these experiments the number of cells produced in the thymus which appeared subsequently in the thoracic duct lymph was small, and the results suggested that the majority of thymus-derived cells did not contribute to the recirculating pool of lymphocytes. Previous experiments with thymectomized fetal lambs (6) indicated that a large proportion of the lymphocytes that make up the mass of fixed lymphoid tissue is derived from the thymus, and these cells cannot be induced to leave the tissues by chronic lymph drainage. The results of the present experiments also demonstrate that most (about 90%) of the lymphocytes that are present in the recirculating pool of cells in the fetus are derived from the thymus or their entry into the recirculating pool is controlled by the thymus. In a 120-day fetus it was calculated that the thymus contributed about 4×10^7 cells/h to the recirculating pool; extrathymic tissue contributed about 4×10^6 cells/h. These values were higher in lambs nearer to term.

The changes in the cell population of the lymph of fetal lambs after continuous

drainage for 2–5 wk were different from those described in rats and mice. Chronic thoracic duct drainage in these species causes an increase in the percentage of large lymphocytes in the lymph and this method was used by Gowans et al. (25, 26) to enrich the cell population of lymph with large lymphocytes. It has also been assumed that the large lymphocytes in normal lymph represent precursor cells in a line of differentiation through large and medium to small lymphocytes (Yoffey and Courtice [27]). There was no indication that the proportion of large cells in the thoracic duct lymph increased during periods of drainage extending over several weeks when newly-formed cells would be expected to enter the recirculating pool at a maximum rate. This held for both normal and thymectomized fetuses. This finding suggested that in the fetus all cells entering the recirculating pool were small lymphocytes even during periods of maximum cell mobilization and production. The results that have been obtained after thoracic duct cannulation for relatively short periods in adult rats, mice, guinea pigs, and calves may reflect differences in the activity of lymphopoietic tissue in these animals in an antigen-charged environment or functional differences in the gut-associated lymphoid tissues at different stages in its development. The intestinal lymph of lambs and sheep normally contains a very high proportion of large lymphocytes of varying size and degrees of basophilia, and these cells are characteristic of lymph from the guts.

The Effect of Chronic Lymph Drainage on the Histology of Fetal Lymphoid Tissues. The lymphoid tissues of the fetal lamb do not have the same histological structure as in lambs after birth. While there is some differentiation between cortex and medulla in fetal lymph nodes and while primary follicles are present, there are normally no germinal centers, and the Peyer's patches are poorly developed until near to birth. This lack of differentiation made it difficult to assess whether so-called thymic-dependent areas, as defined in other species (28, 29), were depleted by chronic drainage. The periarteriolar areas of the spleen were slightly depleted of lymphocytes by thoracic duct drainage, but in the lymph nodes it was equivocal as to whether any one particular area was depleted of lymphocytes more than another even after several weeks of drainage. In fact, it was remarkable how small an effect the removal of vast numbers of free-floating cells had on the content of lymphocytes in the lymphoid tissues of the fetus. The explanation of this may lie in the fact that lymphopoiesis in the fetus is proceeding at a much more rapid rate than in adult animals, and in these circumstances drainage has a differential effect on the fixed and circulating pools of lymphocytes.

Thoracic duct drainage in rats and mice for a period of 5 days severely depletes the lymphoid tissues of small lymphocytes, particularly in the cortical and cortico-medullary regions. Sprent (12) reported that drainage of thoracic duct lymph from adult mice for 5 days led to a depletion of thymus-derived cells in the lymph, in the paracortex of lymph nodes, and in the periarteriolar regions in the spleen. The numbers of small lymphocytes in the interfollicular areas of the Peyer's patches were rapidly depleted by drainage, and the lymphocytes in the primary follicles of lymph nodes were eliminated more gradually. The fact that these changes did not occur in fetal lambs even after weeks of drainage was probably due to the continued replacement of mobilized lymphocytes by newly-

formed cells. However, another explanation of these results should be considered. Yoffey et al. (30) described a striking reduction in the number of small lymphocytes in the cortical areas of the thymus in young rats after only 48 h of thoracic duct drainage. A similarly severe reduction in the numbers of cells in the thymic cortex of calves undergoing thoracic duct drainage has also been reported (31). It was difficult to discern any differences between the histological appearance of thymuses from control fetuses or from fetuses that had been drained for periods of up to 5 wk, and this fact suggests the possibility that the results of studies made on animals after birth may be confounded by other factors that influence the outcome of chronic lymph drainage. Indeed, Fish et al. (31) cautioned that the pronounced histological changes that occurred in the thymuses of calves undergoing thoracic duct drainage might not be due simply to removal of lymphocytes, and the same could be said of changes occurring in other lymphoid tissues. Certainly the conditions under which chronic thoracic duct drainage is carried out in animals after birth are not conducive to the maintenance of a normal physiological status.

In thymectomized fetuses the lymphoid tissues were significantly reduced in size and in their lymphocyte content. It was again difficult to say whether thoracic duct drainage altered the distribution of residual cells in the tissues or whether it caused any significant further reduction in the number of lymphocytes fixed in the tissues. There may have been fewer cells in the lymphoid tissues of the chronically drained thymectomized fetuses, but the most striking fact was just how difficult it was to detect differences between the control thymectomized fetuses and fetuses drained for 3 days or 2 wk. Thymectomy had a far greater effect on the lymphocyte content of lymphoid tissues than did thoracic duct drainage.

Lymphocyte Recirculation in the Fetus. The experiments reported here are the first to show directly that lymphocyte recirculation is established during the early stages of fetal development; lymphocyte recirculation is thus not conditioned by antigenic stimulation or by circulating immunoglobulins. The finding has implications in interpreting the immunological significance of lymphocyte traffic in the initiation and propagation of the immune response. Clearly in the fetus the recirculating cells cannot be memory cells nor can their pathway of recirculation be directed by any immunological stimulus. Recirculation is thus a physiological property of immunologically virgin lymphocytes, and pathways of recirculation must be determined by inherent properties of the cells themselves or the tissues through which lymphocyte traffic occurs. The accumulation of infused labeled lymphocytes in the Peyer's patches and mesenteric lymph nodes cannot be seen to be directed by foreign antigens absorbed from the gut and some other explanation of a more general physiological nature must be sought. It was significant that labeled recirculating lymphocytes left the blood and passed through the interfollicular areas of the Peyer's patches without entering the follicles. In the mesenteric nodes, however, labeled recirculating cells congregated in the follicles. This finding indicated that the cells comprising the follicles in Peyer's patches have a different origin from the cells making up the primary follicles of lymph nodes, and because of this they may well have different potentialities and life histories.

It seems that those lymphocytes that remain in the fetal lamb after thymec-
tomy continue to undergo recirculation from blood to lymph. It has been shown
that in thymectomized rats and mice the lymphocytes that remain after thymec-
tomy recirculate more slowly (12, 32, 33), and this also seems to be the case in
the sheep fetus. Whether this is due to intrinsic differences between different
populations of lymphocytes or due to differences in the kinetics of cell traffic
through the lymphoid tissues of thymectomized animals is not known nor is it
known if this slower rate of recirculation of lymphocytes persists in lambs
thymectomized in utero after they have grown and their lymphoid tissues have
become reconstituted with lymphocytes derived from outside the thymus. This is
something that needs to be decided by further experiments.

Summary

The production and the circulation of lymphocytes has been examined in the
sheep fetus where neither foreign antigen nor immunoglobulins occur. It was
found that as the lymphoid organs increased in size during fetal life, the
numbers and the output of lymphocytes in the thoracic duct lymph increased.
The recirculating pool of lymphocytes was estimated to be $5.5\pm1.5 \times 10^8$ cells in
fetal lambs 95–100 days of age, $5.7\pm1.2 \times 10^9$ cells in fetuses 130–135 days of age,
and $1.2\pm0.3 \times 10^{10}$ cells in fetuses near to term. The rate of addition of
lymphocytes to the recirculating pool was $3.2\pm1.9 \times 10^6$ cells/h in fetuses of 100
days and $3.4\pm0.9 \times 10^7$ cells/h in fetuses of 130 days of age. Lymphocytes
recirculated from blood to lymph in fetuses; labeled cells injected into the blood
stream reappeared in the thoracic duct lymph promptly and reached maximum
levels around 12–18 h after they were injected. Labeled lymphocytes were
detected subsequently in greatest numbers in the lymph nodes, particularly in
the mesenteric lymph nodes and in the interfollicular areas of the Peyer's
patches.

Chronic drainage of thoracic duct lymph from fetuses in utero for periods of up
to 36 days had no obvious effects on the growth or development of the fetus and
only minimal effects on the content of lymphocytes in the various lymphoid
tissues even though the number of cells in the blood and lymph were reduced to
between 20–30% of normal levels.

Thymectomy done in fetuses about 2 mo before cannulation of the thoracic
duct reduced the output of cells in the thoracic duct to about 25% of normal levels
and caused a significant reduction in the content of lymphocytes in the various
lymphoid tissues. Thymectomized fetal lambs subjected to thoracic duct drain-
age for periods up to 2 wk in utero had a similar complement of lymphocytes in
their lymphoid tissues to intact thymectomized fetal lambs. Lymphocytes ob-
tained from the thoracic duct lymph of lambs thymectomized 2 mo previously
recirculated from blood to lymph when they were injected intravenously, al-
though they did this at a significantly slower rate than did lymphocytes from
normal lambs.

References

1. Silverstein, A. M., J. W. Uhr, K. L. Kraner, and R. J. Lukes. 1963. Fetal response to
   antigenic stimulus. II. Antibody production by the fetal lamb. *J. Exp. Med.* 117:799.
2. Silverstein, A. M., and R. A. Prendergast. 1970. Lymphogenesis, immunogenesis,

and the generation of immunologic diversity. *In* Developmental Aspects of Antibody Formation and Structure. J. Sterzl and I. Riha, editors. Academia Publishing House of Czechoslovak Academy of Sciences, Prague.

3. Niederhuber, J. E., D. Shermeta, J. G. Turcotte, and P. W. Gikas. 1971. Kidney transplantation in the fetal lamb. *Transplantation (Baltimore)*. 12:161.
4. Richardson, M., G. H. Conner, C. C. Beck, and D. T. Clark. 1971. Prenatal immunization of the lamb to brucella; secondary antibody response *in utero* and at birth. *Immunology*. 21:795.
5. Fahey, K. J., and B. Morris. 1974. Lymphopoiesis and immune reactivity in the foetal lamb. *Ser. Haematol*. 7:548.
6. Cole, G. J., and B. Morris. 1971. The growth and development of lambs thymectomized *in utero*. *Aust. J. Exp. Biol. Med Sci*. 49:33.
7. Smeaton, T. C., G. J. Cole, M. W. Simpson-Morgan, and B. Morris. 1969. Techniques for the long-term collection of lymph from the unanaesthetized foetal lamb *in utero*. *Aust. J. Exp. Biol. Med. Sci*. 47:565.
8. Hulet, C. V. 1972. A rectal-abdominal palpation technique for diagnosing pregnancy in the ewe. *J. Anim. Sci*. 35:814.
9. Morris, B. 1973. Effect of thymectomy on immunological responses in the sheep. *Contemp. Top. Immunobiol*. 2:39.
10. Dineen, J. K., and D. B. Adams. 1970. The effect of long-term lymphatic drainage on the lymphomyeloid system in the guinea pig. *Immunology*. 19:11.
11. Fish, J. C., A. T. Mattingly, S. E. Ritzmann, H. E. Sarles, and A. R. Remmers. 1969. Circulating lymphocyte depletion in the calf: effect on blood and lymph lymphocytes. *Arch. Surg*. 99:664.
12. Sprent, J. 1973. Circulating T and B lymphocytes of the mouse. I. Migratory properties. *Cell Immunol*. 7:10.
13. Gowans, J. L. 1957. The effect of continuous reinfusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanaesthetized rats. *Br. J. Exp. Pathol*. 38:67.
14. McGregor, D. D. 1966. Studies by thoracic duct drainage of the functions and potentialities of the lymphocyte. *Fed. Proc*. 25:1713.
15. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. R Soc. Lond. B Biol. Sci*. 159:257.
16. Ernström, U., L. Gyllensten, and B. Larsson. 1965. Venous output of lymphocytes from the thymus. *Nature (Lond.)*. 207:540.
17. Larsson, B. 1966. A quantitative estimation of the venous output of lymphocytes from the thymus in guinea pigs. *Acta Pathol. Microbiol. Scand*. 67:586.
18. Williams, M., A. D. Chanana, E. P. Cronkite, and B. H. Waksman. 1971. Antigenic markers on cells leaving calf thymus by way of the efferent lymph and venous blood. *J. Immunol*. 106:1143.
19. Sainte-Marie, Guy. 1973. Cell migration and the thymus. *Contemp. Top. Immunobiol*. 2:111.
20. Kotani, M., K. Seiku, A. Yamashita, and I. Horii. 1966. Lymphatic drainage of the thymocytes to the circulation in the guinea pig. *Blood*. 27:511.
21. Joel, D. D., A. D. Chanana, and E. P. Cronkite. 1974. Thymus cell migration. *Ser. Haematol*. 7:464.
22. Chanana, A. D, E. P. Cronkite, D. D. Joel, R. M. Williams, and B. H. Waksman. 1971. Migration of thymic lymphocytes: Immunofluorescence and ^{3}HTdR labeling studies. *In* Morphological and Functional Aspects of Immunity. K. Lindahl-Kiessling, G. Alm, and M. G. Hanna, Jr., editors. Plenum Publishing Corp., New York. 113.

23. Goldschneider, I., and D. D. McGregor. 1968. Migration of lymphocytes and thymocytes in the rat. II. Circulation of lymphocytes and thymocytes from blood to lymph. *Lab. Invest.* **18**:397.

24. Miller, J. F. A. P., and J. Sprent. 1971. Thymus-derived cells in mouse thoracic duct lymph. *Nat. New Biol.* **230**:267.

25. Gowans, J. L., B. M. Gesner, and D. D. McGregor. 1961. The immunological activity of lymphocytes. *In* Biological Activity of the Leucocyte. Ciba Foundation Study Group 10. Churchill Ltd., London. 32.

26. Gowans, J. L. 1962. The fate of parental strain small lymphocytes in F₁ hybrid rats. *Ann. N. Y. Acad. Sci.* **99**:432.

27. Yoffey, J. M., and F. C. Courtice. 1970. Lymphatics, lymph, and the lymphomyeloid complex. Academic Press, Inc., New York.

28. Parrott, D. M. V., M. A. B. de Sousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. Exp. Med.* **123**:191.

29. de Sousa, M. 1971. Kinetics of the distribution of thymus and marrow cells in the peripheral lymphoid organs of the mouse: ecotaxis. *Clin. Exp. Immunol.* **9**:371.

30. Yoffey, J. M., W. J. C. C. Rich, M. K. Tidman, B. H. Cummins, and R. R. Roy. 1964. The source of lymphocytes in thoracic duct lymph during prolonged drainage. *Ann. N. Y. Acad. Sci.* **113**:1053.

31. Fish, J. C., G. Beauthard, H. E. Sarles, A. R. Remmers, Jr., and S. E. Ritzmann. 1970. Circulating lymphocyte depletion: effect on lymphoid tissue. *Surgery (St. Louis).* **67**:658.

32. Ford, W. L., and S. J. Simmonds. 1972. The tempo of recirculation of lymphocytes from blood to lymph in the rat. *Cell Tissue Kinet.* **5**:175.

33. Howard, J. C. 1972. The life-span and recirculation of marrow-derived small lymphocytes from the rat thoracic duct. *J. Exp. Med.* **135**:185.