white mutation. None of the several thousand females that we examined ever exhibited this abnormal accumulation of drosopterins, and furthermore, even some of the males did not exhibit the phenotype. However, outcrosses to various wild stocks, including Oregon-R and Canton-S, failed to remove the red fat body phenotype. Accumulation of various pteridines in the testes, but not in the ovaries, has been discussed as a secondary sex characteristic in *Drosophila* (*2*).

At first it appeared that those males that developed this pigment did so in the 2nd or 3rd day of adult life. To determine the time of pigment development the following was done: Culture bottles containing 30 pairs of w^{mo} adults were set up at 23°C, and adults were allowed to lay eggs for 1 day. (All flies were raised on cornmeal-yeast-agar medium.) Adults that emerged from these cultures within each 24-hour period were collected and observed daily. Any male that showed visible red pigment in the abdomen was removed from the collection and observed daily for disappearance of the pigment (Fig. 4). Males that emerged during the 1st day never accumulated drosopterins in the fat bodies; those that emerged later, however, did show red deposits in the abdomens. It seems that the later the adults emerged—the longer the developmental period—the sooner the males showed red pigment. The increased developmental period also corresponds with an increased frequency of phenotypic expression. Furthermore, the day-5 males lost the pigment earlier.

In many of the males that were dissected during the time of pigment loss, dark red-brown depositions were seen in the Malpighian tubules. Since the Malpighian tubules reputedly serve as excretory organs (*4*), it is possible that drosopterins or their breakdown products are being excreted through the intestinal tract (*5*). Furthermore, on emergence the fly contains two types of abdominal fat cells (*6*). Disappearance of the pigment corresponds quite well to disappearance of the "pupal-fat" cells.

Protein granules are apparently necessary for normal pigment deposition in the eyes of several insects (*2*). Goldsmith and Kramer (*7*) have described large protein granules in unpigmented *D. melanogaster* fat body, but these are much larger than those found in eyes. Rizki (*8*) has reported the accumulation of ommochromes in the anterior fat body of *Drosophila*. He did not, however, report finding protein granules in this organ. Phase microscopy of living fat body from w^{mo} individuals showed no granules in the cytoplasm.

Testes and Malpighian tubules accumulate drosopterins in some mutants and species of *Drosophila* (*9*). Fat bodies, whose function has been likened to vertebrate liver (*10*), may also play a role in pteridine metabolism.

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Lymphocytes from Thymectomized Rats: Immunologic, Proliferative, and Metabolic Properties

Abstract. *Thoracic duct lymphocytes from neonatally thymectomized Lewis rats fail to produce runt disease in newborn Brown Norway rats when injected with up to ten times the number of normal lymphocytes needed to cause runting. The immunologically deficient lymphocytes appear, however, to confer tolerance, and at least some enlarge and divide when stimulated in vitro with phytohemagglutinin, or xenogeneic or allogeneic cells. Small lymphocytes from thymectomized animals have defective RNA metabolism as judged by a marked impairment in their ability to incorporate uridine-5-H^3 or cytidine-H^3 in vitro.*

The immunologic defects which develop in rats thymectomized at birth have been considered to arise from a failure of central immune mechanisms, such as cell proliferation and interaction, which may be dependent upon thymic function in vivo (*1*). However, it has also been demonstrated that isolated spleen cells from thymectomized mice are in themselves immunologically deficient, for they exhibit a decreased capacity to produce runt or graft-versus-host disease when transfused to normal, allogeneic recipients (*2*). Because the latter work indicated that lymphocytes from thymectomized rodents may be qualitatively as well as quantitatively deficient, it seemed of value to confirm and extend the study in the rat where techniques for lymphocyte culture might permit a further characterization of the nature of the cellular disorder. Accordingly, I describe attempts to induce runt disease and immunological tolerance in Brown Norway (BN) rats by injections of thoracic duct lymphocytes from neonatally thymectomized Lewis rats. Certain proliferative and metabolic characteristics of the immunologically deficient lymphocytes are described as they were observed in short-term tissue culture.

In studies such as this, where identification of the specific cell type involved in immune reactions is important, thoracic duct lymph offers an advantage over spleen or other lymphoid cell suspensions in that it contains lymphocytes in nearly pure population. Because of this, and because thoracic duct lymph yields immunologically active cells (*3*), it was collected either from the neck (*4*) or abdomen (*5*) of donor rats and used throughout the study. Lewis rats (less than 1 day old) were thymectomized by splitting the sternum and dissecting the thymus free after the animals had been chilled at −10°C until they were apneic (5 to 10 minutes). Control animals were either sham-operated or, more commonly, untreated. After serving as cell donors, all thymectomized rats were autopsied to assure the completeness of thymectomy.

The capacity of lymphocytes from neonatally thymectomized Lewis rats to induce runt disease in newborn BN animals was studied first. Thoracic duct cells from thymectomized donors were washed in Hanks balanced salt solution, and, after being resuspended in 0.1 to 0.3 ml, were administered in doses of 1 to 20 × 10^6 lymphocytes per recipient. All injections were given into

the retro-orbital branch of the facial vein of BN animals which were less than 1 day old. Control BN animals received similar injections of cells from nonthymectomized donors. In all, eight thymectomized donors ranging in weight from 100 to 165 g (1½ to 2½ months old) were used to inject 25 neonatal BN recipients. Similarly, 11 nonthymectomized Lewis donors weighing from 160 to 240 g (2 to 3 months old) were used to transfuse 35 newborn control BN rats, many of which were littermates to the animals that received thymectomized Lewis cells. Donors and recipients in experimental and control groups were of both sexes. The results (Table 1) show that while 100 percent of the BN animals which received normal lymphocytes developed fatal runt disease, none of the animals given equal doses of cells from thymectomized donors was afflicted. Experimental animals were weighed and observed daily up to 50 days of age and some have been observed for as long as 5 months. None of these gave evidence of any of the symptoms of runting described for rats (*6*), and the animals could not be distinguished by weight or appearance from noninjected litters. In contrast, the mean time of death for control animals was 17 to 18 days, the range being 14 to 37 days.

Some assessment of the magnitude of the immunologic defect in lymphocytes from thymectomized donors may be gained from Table 1 where it is seen that while as few as 2×10^6 normal thoracic duct cells uniformly produced runt disease, up to 10 times as many cells from thymectomized donors were without effect. This factor may be expressed even more pointedly in terms of small lymphocytes (rather than total cells) where it remains slightly greater than nine even though differential counts of thoracic duct cells in thymectomized animals were shifted away from the small lymphocyte (for thymectomized rats: 85 percent small; 15 percent large and medium; for normal rats: 95 percent small, 5 percent large and medium). Dalmasso *et al.* (*2*) reported that a few mice developed runt disease when treated with spleen cell suspensions from thymectomized donors, and it may also have been possible to induce some runting in the present system if larger numbers of animals or larger cell doses had been used. However, the failure to produce runting by the transfusion of up to nine times as many small lymphocytes from thymectomized rats as needed from control animals to induce lethal disease, provides direct evidence for the indication (*2*) that the lymphocytes which persist after thymectomy are qualitatively abnormal. The mechanism of the immunologic defects which attend thymectomy seems therefore to be more than only a quantitative one relating to lymphopenia. Very recently Osoba (*7*) has drawn attention to the qualitative fault in thymectomized mice by reporting that thymus grafts contained in Millipore chambers will reverse the immunologic defects in the host without correcting the lymphopenia or restoring the tissue content of small lymphocytes.

Table 1. Incidence of lethal runt disease in newborn BN rats transfused with thoracic duct lymphocytes from normal and neonatally thymectomized Lewis rats.

Number of cells transfused $\times 10^6$	Recipients (No.)	Lethal runting (No./total)
Normal Lewis donors		
2–2.5	14	14/14
5	14	14/14
7–10	5	5/5
15–20	2	2/2
Totals	35	35/35*
Thymectomized Lewis donors		
1	2	0/2
5	8	0/8
7–10	9	0/9
15–20	6	0/6
Totals	25	0/25†

* 100 percent. † Zero percent.

The failure of lymphocytes from thymectomized Lewis animals to induce runt disease in neonatal BN rats led to the consideration of whether these cells also were incapable of producing immunological tolerance. The question was whether the adult BN rats which had been injected as newborns with cells from thymectomized Lewis had circulating lymphocytes that were tolerant to Lewis cells. To assess this, attempts were made to produce runt disease in newborn Lewis animals by injecting them with thoracic duct lymphocytes from previously transfused, adult BN rats. Five 30- to 50-day-old animals which as neonates had received 5 to 20 $\times$ 10^6 lymphocytes from thymectomized Lewis rats were used to transfuse newborn Lewis recipients. Six were given 5×10^6 lymphocytes, and four others received 10×10^6 cells each. Four control BN animals, comparable in age to the experimentals but which had not received Lewis cells, were used to transfuse four newborn Lewis with 5×10^6 BN cells, and four others with 10×10^6 lymphocytes each. The results showed that all Lewis animals that received lymphocytes from uninjected (control) BN donors developed definite signs of runt disease by the 17th day of life, and, by day 22, five of the total of eight had died. The remaining three belonged to the group which received the lower dose of cells (5×10^6), and they remained symptomatic but did not die. In contrast, of the ten animals which received lymphocytes from previously transfused BN donors, nine of the ten gained weight normally and showed no signs of runting. Only one of the ten developed runt disease and died at day 18. These findings indicated that immunological tolerance could be conferred by lymphocytes from thymectomized animals, and, in addition to confirming the report by Dalmasso *et al.* (*2*) in this regard, pointed to an interesting dissociation between the mechanisms by which lymphocytes confer tolerance or produce runt disease. This dissociation has been widely recognized when embryonic or neonatal (immunologically incompetent) cells have been employed to induce tolerance without causing runting, and it may be that lymphocytes in adult rats which have been thymectomized at birth should be viewed as being "embryonic" in their immunologic capabilities.

Small lymphocytes initiate an immune response to genetically dissimilar tissue by enlarging and dividing (*3, 8*). It seemed not impossible, therefore, that the failure of lymphycytes from thymectomized animals to mount an effective graft-versus-host reaction might arise from an inability of these cells to proliferate in response to foreign stimuli. To test this possibility, lymphocytes from thymectomized animals were maintained in short-term tissue culture (*9*), and were stimulated with a variety of mitogenic agents. The mitogenic stimuli were tested in a series of increasing specificity in relation to the graft-versus-host reaction by evaluation first of the responsiveness of thymectomized Lewis lymphocytes to phytohemagglutinin-P and then to xenogeneic (mouse) cells and finally allogeneic (BN) lymphocytes.

Cells of 15 adult Lewis rats which had been thymectomized at birth were used to establish 18 cultures containing 3 to 4 $\times$ 10^6 thoracic duct lymphocytes per milliliter and phytohemagglutinin-P in media exactly as previous-

ly reported (*9*). After 2 or 3 days at 37°C the cultures were killed, and smears were prepared. Some of the donor animals had previously been injected with thymidine-H^3; smears from cultures containing these cells were dipped in Eastman NTB_3 photographic emulsion for radioautography.

All cultures grew in response to phytohemagglutinin-P and contained up to 25 percent blast forms by day 3, with up to 1 percent mitotic figures. While some cells in all cultures clearly responded to phytohemagglutinin-P, the magnitude of the response varied among cultures, and the total cell number at day 3 was commonly one-half or less that obtained from comparable cultures of normal thoracic duct lymphocytes. At least some of the blast forms in cultures of lymphocytes from thymectomized rats came from small lymphocytes, as shown by culturing thoracic duct cells from two thymectomized rats that had had daily intraperitoneal injections of thymidine-H^3 (1 μc per gram of body weight, specific activity 6.7 c/mmole) for 14 days and then were rested for 14 days without further isotope. When radioautographs of lymphocytes from these animals were limited to a 3-week film exposure the smaller lymphocytes were the only cells labeled with more than three silver grains, and no cell with nuclear diameters greater than 10 by 10 μ qualified as being "labeled" by that standard (Fig. 1). While none of the large cells were labeled in the culture inoculum, after 2 days of phytohemagglutinin-P stimulation up to 10 percent of the blasts evidenced between 5 and 40 grains over their nuclei (Fig. 2). Cultures of this sort were grown with excess nonradioactive thymidine (4.8 μg/ml or 125 times as much thymidine as would be present in 1 μc of thymidine-H^3 per milliliter) to prevent possible reutilization by the blasts of any radioactive thymidine released by the death of labeled small cells.

With the demonstration that lymphocytes from thymectomized rats responded to phytohemagglutinin P, attention was given to whether similar enlargement and division would occur when rat cells were grown with the somewhat more specific stimulus provided by xenogenic tissue. Accordingly, four cultures were established in media used for phytohemagglutinin-P cultures except that the phytohemagglutinin was omitted. Two of the cultures contained 4.5×10^6 lymphocytes per milliliter from a thymectomized Lewis rat and two contained the same concentration of lymphocytes from a normal rat. A mixture of spleen and mesenteric lymph node cells taken from an adult Ajax mouse was added to each culture in a concentration of 5.2×10^6 cells per milliliter. After 2 or 3 days incubation, colchicine (0.1 μg/ml) was added to the cultures, and 3 hours later chromosome spreads were prepared (*10*). The difference in karyotype between the mouse and rat (Fig. 3) permitted a simple and positive identification of

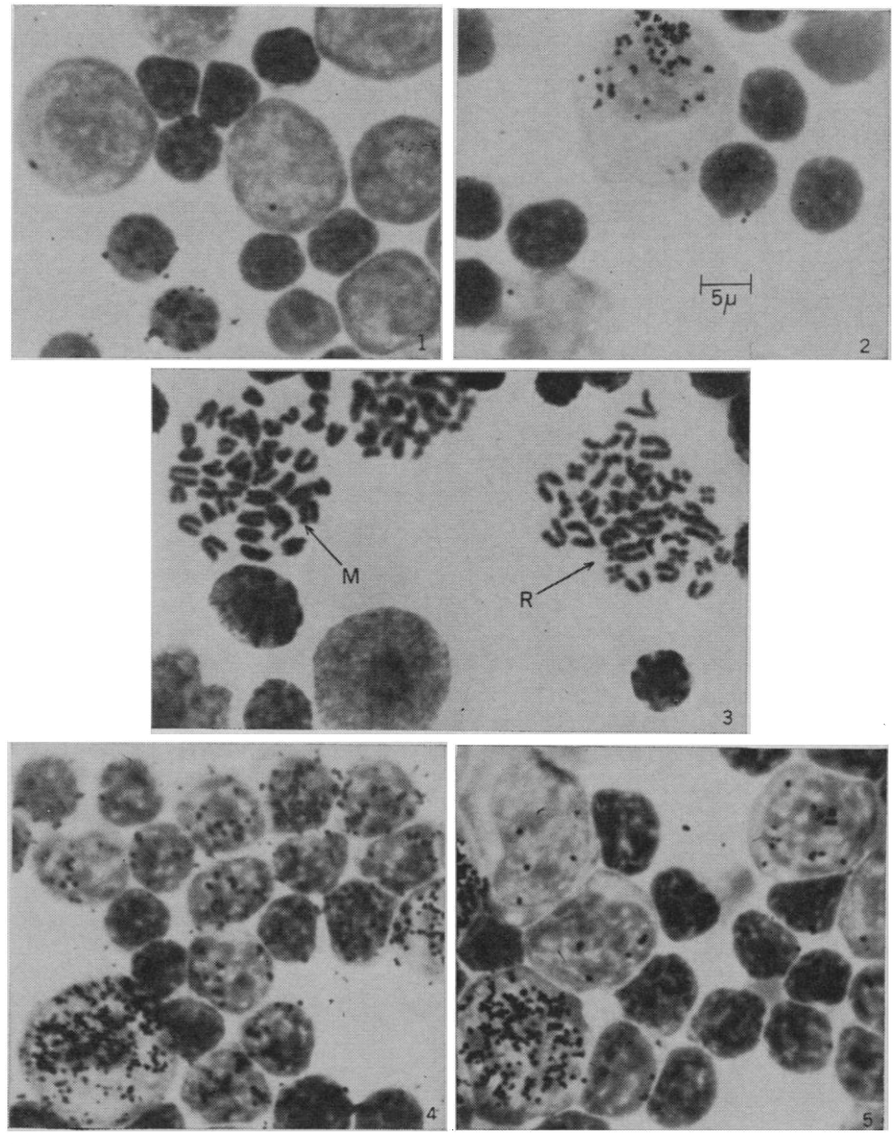

Fig. 1. Radioautograph of thoracic duct lymphocytes from a neonatally thymectomized Lewis rat which had received daily thymidine-H^3 injections for 2 weeks, 14 days before being killed. These cells which show radioactivity only in small lymphocytes were used in cultures with phytohemagglutinin.

Fig. 2. Radioautograph of cells in a 2-day culture of the lymphocytes shown in Fig. 1 with phytohemagglutinin. The labeled blast cell has arisen from a labeled small lymphocyte.

Fig. 3. Metaphase spreads of a mouse (*M*) and rat (*R*) lymphocyte are shown from a 2-day culture of Lewis rat lymphocytes mixed with a suspension of spleen and lymph node cells from an Ajax mouse. The mitogenic stimulation afforded by mixtures of xenogeneic cells was effective with either normal lymphocytes or those from thymectomized rats.

Fig. 4. Radioautograph of thoracic duct lymphocytes from a normal Lewis rat after the cells had been incubated 1 hour with 10 μc of uridine-5-H^3 per milliliter. Nearly all cells are labeled.

Fig. 5. Radioautograph of thoracic duct lymphocytes from a neonatally thymectomized Lewis rat after the cells had been incubated 1 hour with 10 μc of uridine-5-H^3 per milliliter. Many small cells have incorporated no isotope.

dividing cells as well as the establishment of the proliferative ratio between the species. All four cultures showed definite growth and 100 metaphase spreads were counted in each. The ratio of rat to mouse mitotic figures in cultures containing lymphocytes from the normal Lewis donor were 73:27 at day 2 and 81:19 at day 3. These ratios in cultures with lymphocytes from the thymectomized donor were 78:22 at day 2 and 79:21 at day 3. The difference in the tissue source of cells between the species does not allow interpretation of the observation that rat mitotic figures were consistently three to four times more numerous than mouse. In addition, the data do not permit the assertion that the responses in experimental and control cultures were quantitatively identical. However, the similarity of the ratios in cultures containing lymphocytes from normal as compared to thymectomized donors argues that at least many lymphocytes from thymectomized rats retain the capacity to enlarge and divide in response to foreign tissue.

The final and most specific test of the capability of lymphocytes to enlarge and divide in the graft-versus-host reaction was conducted by culturing lymphocytes from thymectomized, thymidine-H^3 labeled, Lewis rats in the presence of lymphocytes from the allogeneic BN strain. Previous studies in this laboratory have established methods and shown that in cultures of lymphocytes mixed from normal donors of the Lewis and BN strains there is a marked blastogeneic response (*11*). Thoracic duct lymphocytes were taken from two thymectomized Lewis rats and two normal BN animals and used to establish 8 cultures. The cell concentration was adjusted so that 2.5×10^6 lymphocytes from each strain were present per milliliter of medium. The Lewis donors had received daily intraperitoneal injections of thymidine-H^3 (1 μc per gram of body weight) for 14 days, the last injection being 20 days before the animals were used. Cultures were incubated for 2, 3, and 4 days and then sacrificed so that smears could be prepared for radioautography. All cultures contained enlarged and dividing cells, and the number of enlarged cells increased from day 2 to day 4 of culture to reach approximately 25 percent of all cells present. Radioautographs of the Lewis lymphocytes used to initiate cultures showed that, while approximately 11 percent of the small cells in the inoculum were radioactive, no large cell was labeled with more than three silver grains. By days 3 and 4 of culture, however, 2 percent of the total enlarged cells were labeled with 6 to 15 grains. Thus it appeared that lymphocytes from the labeled, thymectomized Lewis donors did respond to the stimulus of allogeneic BN cells and that at least some of the blast cells arose from small lymphocytes. The fact that the percentage of labeled blasts was considerably less than found when labeled lymphocytes were cultured with phytohemagglutinin-P was interpreted to mean that many of the blasts in mixed lymphocyte cultures had derived from unlabeled BN cells which had been stimulated by the thymectomized Lewis cells. Relative to all of the studies in vitro with phytohemagglutinin-P or foreign cells, control cultures of thoracic duct lymphocytes without phytohemagglutinin-P or foreign cells never showed more than 1 percent enlarged cells in the intervals employed (*9, 11*).

The studies in vitro on the proliferative potential of lymphocytes from thymectomized rats showed that their immunologic impairment could not be attributed to a total inability to recognize foreign tissue or to proliferate in response to it. However, the proliferative response may have been defective both in terms of the number and of the metabolic (and possibly, therefore, the immunologic) capabilities of the responding cells. The low cell number in phytohemagglutinin-P cultures suggested that fewer than the normal number of cells responded to mitogenic agents, and the observation that enlarging blast forms in culture were often less basophilic than normal indicated that they might have a disorder in their RNA metabolism. To investigate the possibility of such a disorder, thoracic duct lymphocytes from four thymectomized and four normal Lewis rats were suspended separately at concentrations of 2 to 10×10^6 cells per milliliter in 5 ml of a mixture of 80 percent Eagle minimum essential medium and 20 percent fresh rat serum. Uridine-5-H^3 or cytidine-H^3 (10 μc/ml) was added to two each of the suspensions from thymectomized and normal rats. After one hour incubation at 37°C the cell suspensions were washed and smears were prepared from each for radioautography. In radioautographs exposed for one week a striking difference was found in relation to the labeling of small cells. While 53 to 92 percent of normal small lymphocytes evidenced 3 to 35 silver grains per cell, only 11 to 18 percent of these cells from thymectomized rats had incorporated a sufficient quantity of either of the ribonucleic acid precursors to be judged labeled, and most of these were much more lightly labeled than their normal counterparts (Figs. 4, 5). Large lymphocytes of the two groups were more equally labeled with 80 to 100 percent of the normal and 80 to 95 percent of the cells from thymectomized rats showing radioactivity. Again, however, lightly labeled large lymphocytes were more frequently seen in cells from thymectomized animals. The apparent defect in RNA metabolism (RNA synthesis and possibly RNA degradation) in small lymphoctyes from thymectomized animals must also be studied in these cells as they undergo an immune reaction. Moreover, a critical demonstration must be made that the defect is causally and not just coincidentally related to the immunologic impairment. However, for the present it seems reasonable to assume that this deficiency reflects a qualitative abnormality in lymphocytes from neonatally thymectomized rats and may, therefore, be associated with their inability to produce runt disease in normal, allogeneic recipients.

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