A knowledge of the normal processes of thymic development is clearly of considerable importance to a full understanding not only of the function role of the thymus in immunological maturation, but also of the pathogenesis of congenital thymic abnormalities. Some abnormal conditions are characterized by a complete absence of the thymus at birth (1); others show small remnants of thymic tissue (2). In either case, analysis of the interrelationships between the primary embryological components of the early thymus should provide a basis for further investigations.

The histogenesis of the early thymic rudiment is, however, still a subject of controversy. While there has been general agreement that the primary anlage is derived from epithelium mainly of the third, but also of the fourth, pharyngeal pouches (3–6), the origin of lymphoid cells (thymocytes) which subsequently proliferate in the rudiment has not been conclusively settled. On the one hand, a considerable number of histological studies on a variety of mammals, birds, reptiles, and fish have affirmed that lymphoid cells arise intrinsically by direct “transformation” of epithelial cells within the rudiment (7–12). On the other hand, equally numerous studies support the derivation of thymocytes from extrinsic cells of mesenchymal origin which migrate into the primitive epithelial anlage at an early stage (13–20). These contradictory interpretations of data, which in many cases were obtained on the same species, illustrate the difficulty in analyzing cellular kinetics by histological methods alone.

Auerbach (21) provided an experimental approach to the problem by separating thymic rudiments of 12 day mouse embryos into epithelial and mesenchymal components. The separate developmental potential of these components was then tested in combinations with nonthymic tissue from mouse and chick embryos maintained in vitro and as transplants. Since lymphoid tissue developed in the epithelial but not the mesenchymal component of the rudiments, Auerbach concluded that the initial lymphoid cell population of the mouse thymus is derived locally by differentiation of epithelial cells and that migrating mesenchymal cells do not contribute significantly. However, this interpretation is critically dependent upon the supposition that all cells in the “epithelial” component of the 12 day rudiment are, in fact, cells of epithelial origin. The possibility that mesenchymal cells migrate into the epithelial component of the rudiment prior to the 12 day stage was not conclusively ruled out.

Various attempts to determine the origin of thymocytes have been made by transplanting early thymic primordia to a variety of hosts and sites (22–25). The main object of these studies was to decide whether or not the grafted rudiments were in-
vaded by host cells. They failed to do so because of the difficulty in distinguishing host from donor cells by histological methods alone.

Recently, a considerable body of data has been obtained from chromosome marker experiments in the adult animal which suggests that migration of stem cells between various hemopoietic organs (including the thymus) is an important factor in hemopoiesis (26, 27). This dynamic concept of adult hemopoiesis contrasts markedly with views of embryonic blood cell formation which presume that stem cells are derived locally from fixed elements in each separate hemopoietic organ. Studies have been designed in this laboratory, therefore, to determine whether there is a pattern of cellular migration in the embryo comparable to that in the adult (28, 29). The results have indicated that there is a considerable inflow of blood-borne cells to the spleen, bone marrow, and bursa of Fabricius during avian development. In the present investigation, the possibility that a similar inflow occurs in the developing thymus has been studied.

The sex chromosome difference between male (ZZ) and female (ZW) avian cells provides an excellent marker system for investigating the interflow of cells between chick embryos joined by a vascular union. Hence, in a first series of experiments, chromosome preparations were made from thymic cells of embryos joined by parabiosis. In a second series, the relative contributions of host and donor cells to the lymphoid development of embryonic thymic grafts made to the chorioallantois were studied by the chromosome marker technique. Finally, a histological investigation was made on chick and mouse thymic rudiments in order to delineate the types of cell present in early organogenesis and to compare the pattern of development in the two species.

**Materials and Methods**

**Chromosomal Analysis.**—Recently, a technique has been developed in this laboratory for the preparation of clear chromosome spreads from various hemopoietic organs of the chick embryo (30). Male (ZZ) and female (ZW) cells can be readily distinguished from each other as the Z chromosome is the only large mediocentric element present. 3 hr before sampling, eggs were injected with 0.1 ml Colcemid solution (0.05-0.1 mg Colcemid according to stage of incubation). Chromosomes were prepared using a modified hypotonic citrate air-drying technique. Staining was carried out in lactic-acetic orcein and the preparations were mounted in Euparal and viewed by phase-contrast microscopy.

**Histological Methods.**—Block dissections were made on chick embryos of 7 and 8 days incubation to remove thymic rudiments together with surrounding tissues. After 24 hr in Bouin’s fixative, the rudiments were embedded in polyester wax and sectioned at 5 μ. Sections were stained in Giemsa, dehydrated in acetone and mounted in De Pe X (G. Gurr, Ltd., New King’s Road, London, England).

Thymic rudiments of F1 mice (CBA.H × C57.BL) of 11 and 12 days gestation were also sectioned. In order to determine the gestation stage accurately, advantage was taken of the fact that mice will mate within a period of 24 hr of delivery of a litter. Up to 85% of animals may conceive during this interval (31). Cages were examined each morning and litters born during the preceding 24 hr were removed. Males were left with females for a further 6 hr and
then they also were removed. Females were killed at exactly 11 and 12 days from the morning of the initial examination.

Parabiosis of Avian Embryos.—Two types of vascular anastomosis were established between embryos. In a previous investigation (28), embryos of 6 days incubation were parabiosed by direct apposition of their chorioallantoic membranes. In the present study, in order to effect a very early vascular union, embryos of 4 days incubation were parabiosed via a yolk sac anastomosis. The technique for producing a yolk sac union was as follows: Eggs were incubated in a fixed horizontal position so that the developing embryo and yolk sac floated to the uppermost part of the egg and albumen to the lowest part. After 4 days’ incubation in this fixed position, the embryo and surrounding blood vessels lay in close apposition to the chorion and shell membrane. A drop of saline was then placed on a small hole made in the shell membrane over this area and, by applying suction to a hole made into the air space, the embryo was "dropped" away from the shell. A hole of 1 cm diameter was cut in the shell and after removing intervening shell membrane, two eggs were carefully brought together so that the chorions and closely attached vascular yolk sacs made contact. The union was sealed with wax and the eggs sampled 8—12 days later. If embryo pairs were of opposite sex, the thymus and various other hemopoietic tissues were prepared for chromosome analysis.

Chorioallantoic Grafting.—Thymic rudiments were cleanly dissected from chick embryos of 8-15 days incubation and were transplanted to the chorioallantoic membrane of 10 day chick embryo hosts using the technique described by Hancox (32). After a further 4-9 days incubation, hosts were injected with Colcemid and 3 hr later grafts were removed, weighed, and, if host and donor were of opposite sex, prepared for chromosome analysis. In addition, a histological examination of graft development was made on rudiments removed at 24 hr intervals after transplantation.

Thymic rudiments of 12 day mouse embryos were carefully dissected out. At this stage, the paired rudiments were clearly visible in the anterior mediastinum and could be cleanly removed. They were then transplanted to the chorioallantois of 10 day chick hosts and after varying intervals they were examined histologically.

RESULTS

The results, to date, of chromosome analysis made on thymus tissue of chick embryos joined by chorioallantoic anastomoses are presented in Table I. With the exception of two embryos sampled at 19 and 20 days after incubation, there is a complete absence of cellular chimerism as judged by the marker technique. However, it is relevant to note that, although embryos were operated on 6-8 days after incubation, a vascular union was probably not effected for a further 2-3 days, when thymic lymphopoiesis was already under way. A vascular union was probably established at a slightly earlier period in twin embryos, and here there was some cellular intermixture within the thymus of all partners (Table I).

In embryos joined by a yolk sac union (Text-fig. 1), and hence having a vascular connection from a very early stage of incubation, high levels of thymic chimerism (up to 70% of the dividing cells being derived from the partner, Table 1) are found. In all cases, at the times of sampling, histological examination of the thymus shows that the vast majority of dividing cells are lymphoid and the over-all results suggest that the degree to which this population is chimeric is critically dependent on the presence of an early vascular union.

Thymic grafts are quickly incorporated into the chorioallantois (Fig. 1) and
histological examination at daily intervals shows that there is good subsequent lymphoid development, the majority of dividing cells at any stage being of this type. While no evidence of necrosis is found in 8–12 day rudiments, it is present in varying amounts in older transplants for a short period after grafting. Thy-

<table>
<thead>
<tr>
<th>Age at sampling</th>
<th>Mitoses of opposite sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td>F</td>
</tr>
</tbody>
</table>

Chorioallantoic union
Pair 1 | 0 | 0 |
Pair 2 | 15 | 0 | 0 |
Pair 3 | 17 | 0 | 0 |
Pair 4 | 0 | 0 | 0 |
Pair 5 | 3 | 3 | 3 |
Pair 6 | 19 | 0 | 0 |
Pair 7 | 12 | 12 | 12 |
Pair 8 | 20 | 12 | 12 |

Twin embryos
Pair 1 | 11 | 8 | 2 |
Pair 2 | 12 | 1 | 4 |
Pair 3 | 13 | 4 | 4 |

Yolk sac union
Pair 1 | 44 | - | - |
Pair 2 | 58 | 8 | 8 |
Pair 3 | 14 | 32 | 4 |
Pair 4 | 52 | 26 | 26 |
Pair 5 | 15 | 6 | 6 |
Pair 6 | 16 | 70 | 70 |

Chromosome analysis made on between 50 to 100 dividing cells in each thymus.
In a number of instances, one partner of a pair was killed by the Colcemid treatment and no chromosome preparations were obtained.

mic growth, particularly in the younger grafts, compares favorably with that in vivo (Table II). Chromosome analysis shows that all grafts are eventually populated by host cells, but the age of the thymic rudiment at the time of transplantation determines the rate at which this occurs (Text-fig. 2). Thus 8 day grafts are almost completely populated by host cells 4 days after transplantation, whereas 10–15 day grafts remain principally donor in type after the same interval, although they are eventually populated by dividing host cells.
The number of host-derived cells in 9 day transplants is very variable at the 4 day stage; in some cases, the dividing cells sampled are almost entirely donor in type and sometimes almost entirely host (Table III).

Text-fig. 1. Embryos joined by a yolk sac anastomosis (arrowed). They were operated on at 4 days incubation and sampled 11 days later. In some embryo pairs there was a partial herniation of yolk sac from one embryo into its partner.

TABLE II

<table>
<thead>
<tr>
<th>Interval</th>
<th>At transplantation</th>
<th>4 days</th>
<th>5 days</th>
<th>6 days</th>
<th>7 days</th>
<th>8 days</th>
<th>9 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 ± 0.24</td>
<td>9.9 ± 1.20</td>
<td>13.2 ± 1.30</td>
<td>16.0 ± 1.04</td>
<td>22.7 ± 2.30</td>
<td>26.5 ± 3.11</td>
<td>27.6 ± 2.70</td>
</tr>
<tr>
<td>Total weight of graft thymus, mg</td>
<td>12.0 ± 1.20</td>
<td>16.5 ± 2.00</td>
<td>28.2 ± 3.10</td>
<td>36.0 ± 5.19</td>
<td>44.7 ± 4.26</td>
<td>56.0 ± 9.21</td>
<td></td>
</tr>
</tbody>
</table>

The means are based on weights of five organs at each stage.

Thymic primordia removed from 12 day mouse embryos and transplanted to the chorioallantois showed lymphoid development which was maximal at 7 days after transplantation, after which the grafts involuted. Mouse and chick cells can be adequately distinguished from each other in tissue sections (33),
Text-Fig. 2. Graph shows the proportion of dividing host cells in chick thymic transplants to the embryo chorioallantois after various intervals. Each point represents a chromosome analysis made on a single transplant. Note that 8 day grafts are almost entirely populated by dividing host cells 4 days after transplantation, whereas at this time 10-15 day grafts are still almost entirely donor in type. The rate of host cell population of 9 day transplants is very variable.

### TABLE III

*Percentage Dividing Host Cells in Chick Thymic Grafts after Various Intervals on the Chorioallantois*

<table>
<thead>
<tr>
<th>Age at grafting</th>
<th>Interval after grafting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 days</td>
</tr>
<tr>
<td>days</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>92, 92, 94, 100, 100</td>
</tr>
<tr>
<td>9</td>
<td>20, 21, 30, 74, 82, 89</td>
</tr>
<tr>
<td>10-11</td>
<td>2, 4, 6</td>
</tr>
<tr>
<td>12-13</td>
<td>2</td>
</tr>
<tr>
<td>14-15</td>
<td>6</td>
</tr>
</tbody>
</table>

Percentages based on 100 dividing cells analyzed from each graft.

and examination of these grafts shows that all lymphoid cells are murine in type (Fig. 2). This confirms the observations of Auerbach (21).

Finally, histological examination of rudiments of mouse embryos of 11-12 day gestation and chick embryos of 8-9 day incubation reveals the presence of two distinct cell types. In both the chick and mouse thymus, after appropriate fixation and staining, cells can be found which are characterized by the presence
of very prominent nucleoli and heavily basophilic cytoplasm. Although few in number in the 11 day mouse thymic rudiment (Figs. 6-8), by 12 days they are numerous and scattered throughout the thymus (Figs. 9-11). At both stages, they can be found in the loose mesenchyme surrounding the rudiment. The 8 day chick rudiment contains cells of exactly the same morphology (compare Figs. 3-5 to Figs. 6-11) and, in terms of their number, this stage is intermediate between the 11 day and 12 day mouse stages. After 9 days of incubation basophilic cells in the chick are very numerous throughout the rudiment. Whatever the derivation of these cells, they are clearly present from, at least, the 11 day stage in the mouse rudiment and the 8 day stage in the chick rudiment.

**DISCUSSION**

The chromosome marker technique was used by us in a previous study to demonstrate high levels of cellular chimerism in the spleen, bone marrow, and bursa of Fabricius of embryos joined by a chorioallantoic union (28). It was concluded that development of these organs is dependent upon an inflow of blood-borne stem cells which proliferate and differentiate within the environment of the organ anlage. Significant degrees of cellular chimerism were not found within the thymus, which suggests either (a) that thymocytes are derived from stem cells intrinsic to the organ anlage (epithelial cells) or (b) that because thymic development begins at a very early stage of incubation, the anlage may have already received a large complement of extrinsic stem cells by the time a chorioallantoic union is effected.

The very high levels of cellular chimerism found in the thymus of embryos joined by an early yolk sac union indicate that the second interpretation is correct. The result strongly suggests that most of the dividing thymus cells at the time of sampling are derived from blood-borne stem cells. Differences in precise timing of yolk sac union and in developmental maturity of embryos at the time of anastomosis may account for the variability in degree of chimerism found.

The fact that grafts of avian thymic anlage made to the chorioallantois are populated by host cells also suggests a derivation of thymocytes from blood-borne stem cells. The greater rate of population of 8 day transplants, as compared to 10 day transplants, suggests that, while older grafts contain sufficient stem cells to produce a mainly donor cell complement 4 days after transplantation, 8 day grafts contain few stem cells and therefore are largely host populated after the same interval. The rate at which 9 day grafts are populated by host cells is very variable and this probably reflects individual graft differences in developmental stage (and therefore stem cell content) at transplantation. Since the grafts show good structural development and growth with no necrosis, the results support the conclusion that there is a considerable stem cell inflow to the normal thymic anlage between 8 and 10 days after incubation.
The significance of host cell population of grafts older than 12 day incubation is complicated by the fact that they undergo some initial necrosis. Consequently, the inflow of host cells may, in part, represent repopulation of damaged tissue. It is interesting to note, however, that the rate of repopulation is constant for grafts of differing age and size.

The absence of an inflow of avian cells into routine thymic grafts confirms the observations of Auerbach (21). However, this does not necessarily imply that there is no stem cell inflow into the normal mouse embryo thymus. It seems more likely that avian stem cells do not enter the murine rudiment because of the wide species difference between donor and host.

The morphological identity of the circulating stem cell has not so far been discussed. For a number of reasons, it seems most likely that the large basophilic cells seen in and around the 8 and 9 day chick thymic rudiments are stem cells. First, they appear within the rudiment at the stage corresponding to the time of major cell inflow as indicated by the chromosome marker experiments. Second, at this stage, large basophilic cells are present within the loose mesenchyme around the thymic rudiment and in blood vessels, and the histological picture could be interpreted as suggesting a cellular migration from the blood stream to the rudiment. Third, experiments carried out in this laboratory\(^1\) show that when tritiated thymidine-labeled cells of embryonic marrow and spleen are injected intravenously into embryos of various ages, hemopoietic organs show a peak uptake of labeled basophilic cells coincident with the initiation of organ hemopoiesis (at 8–10 days incubation in the thymic anlage).

The close similarity between the histological picture in chick and mouse thymic rudiments suggests that a similar process of stem cell inflow may take place in both. Although there may be developmental differences between mouse embryos of the same gestation time, it seems likely that with appropriate staining the 12 day rudiments used by Auerbach (21) may have shown basophilic cells of the type demonstrated at both the 11 and 12 day stages in this study. If these basophilic cells are stem cells, their presence within the epithelial anlage could fully account for its subsequent lymphoid differentiation in vitro. Thus the epithelial component of the rudiment, rather than producing lymphoid cells itself as proposed by Auerbach, may furnish a specific inductive environment which determines the proliferation and differentiation of the basophilic stem cells derived extrinsically. It is interesting to note that the differentiation of the epithelium in turn is dependent upon an inductive influence of adjacent mesenchyme (34).

\(^1\)Moore, M.A.S., and J.J.T. Owen. Unpublished observations.
result from a deficiency in one of these primary embryological components alone. This analysis should provide a basis for further studies.

Finally, the results of this and previous chromosome marker studies on embryonic hemopoiesis allow the following general conclusions:

(a) There is an inflow of blood-borne cells into the primary lymphoid organs, namely, the thymus and bursa of Fabricius, which takes place during early organogenesis. In both cases, stem cells migrate into an epithelial environment and it is proposed that the interaction between the two is responsible for the structural and functional differentiation of the organs. The development of the spleen and bone marrow is also dependent upon an inflow of stem cells, but into a mesenchymal environment.

(b) The stem cells are characterized by a heavily basophilic cytoplasm, prominent nucleolus, and irregular outline.

(c) The most likely primary source of stem cells is the area vasculosa and subsequently the yolk sac. The yolk sac is the only major site of production of blood cells when the thymus is first developing, and experiments using the chromosome marker technique in irradiated chick embryos show that yolk sac cells are capable of populating all hemopoietic tissues of the embryo, including the thymus and bursa of Fabricius.

(d) Although no direct evidence has been presented that the circulating stem cell has unlimited potentialities for hemopoietic development, the results are fully compatible with the hypothesis that it is initially uncommitted and that its subsequent differentiation into particular blood cell types depends upon the nature of the environment of the organ anlage in which it proliferates.

The concept of a dynamic cellular interflow in embryonic hemopoiesis is fully compatible with the evidence for cellular migration in the adult mouse obtained by similar techniques, and it strongly suggests that these mechanisms are basic to hemopoietic development.

SUMMARY

Experiments utilizing chromosome marker and histological techniques in combination with parabiosis and transplantatation procedures have demonstrated an inflow of blood-borne stem cells into the chick embryo thymic rudiment. There is close similarity between the histological picture in the chick and in the mouse thymic rudiment, and it is proposed that a similar developmental process takes place in both. It may be concluded that the epithelial component of the thymic rudiment, rather than producing lymphoid cells itself, furnishes an inductive environment for the proliferation and differentiation of stem cells derived extrinsically.

BIBLIOGRAPHY


EXPLANATION OF PLATES

PLATE 51

Fig. 1. Chick thymic rudiment (removed from a 10 day embryo) after 48 hr on the chorioallantois. Note the absence of necrosis and presence of lymphoid differentiation in the graft. Hematoxylin and trichrome stains. × 800.

Fig. 2. Mouse thymic rudiment (removed from a 12 day embryo) after 7 days on the chick embryo chorioallantois. The cells of the graft are murine in type (they have more dark-staining nuclei and more prominent nucleoli than chick cells). Hematoxylin and trichrome stains. × 1300.
(Moore and Owen: Development of thymus)
PLATE 52

Fig. 3. Section of the thymic rudiment of a normal 8 day chick embryo. The rudiment is separated from surrounding mesenchyme by a distinct basement membrane. There are cells with dark-staining cytoplasm (basophilic) within the rudiment and one is arrowed in the mesenchyme. Giemsa stain. × 960.

Fig. 4. High power view of basophilic cell within the rudiment shown in Fig. 3. The basement membrane separating epithelium from mesenchyme is arrowed. Giemsa stain. × 3000.

Fig. 5. Further high power views of basophilic cells within rudiment shown in Fig. 3. Basement membrane arrowed. Giemsa stain. × 3000.
PLATE 53

Fig. 6. Thymic rudiment of 11 day mouse embryo. Two heavily basophilic cells are arrowed; one is just within the rudiment, the other in the surrounding mesenchyme. Giemsa stain. × 960.

Fig. 7. High power view of basophilic cell within the mouse rudiment shown in Fig. 6. The cell can be clearly distinguished from surrounding epithelial cells by both its heavily staining cytoplasm and the size and depth of staining of its nucleoli. Giemsa stain. × 3000.

Fig. 8. This is also a high power view of an 11 day mouse embryo rudiment. A basophilic cell lies just within the pale-staining epithelial cells of the rudiment (E). Note the surrounding mesenchyme (M). Giemsa stain. × 3000.
(Moore and Owen: Development of thymus)
Fig. 9. Thymic rudiment of 12 day mouse embryo. The outline of the rudiment is arrowed. By this stage there are large numbers of basophilic cells within it. The cell (b) is shown at higher magnification in Fig. 10. Giemsa stain. × 960.

Fig. 10. This high power view shows the characteristic features of basophilic cells within the 12 day mouse rudiment. Their cytoplasm is finely granular (the granules are probably polyribosomes) and in some cells a prominent juxtanuclear vacuole is present (probably the Golgi structure). Note the similarity between these cells and those seen in the 8 day chick rudiment Figs. 4 and 5). Giemsa stain. × 3000.

Fig. 11. Section of another 12 day mouse thymic rudiment. Note the large number of basophilic cells within it. Giemsa stain. × 960.
(Moore and Owen: Development of thymus)