TISSUE INTERACTION IN THE DEVELOPMENT OF THYMUS LYMPHOCYTES

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Recently, considerable interest has been shown in the pathogenesis of immunological deficiency diseases, especially in associations between developmental anomalies of primary lymphoid organs and functional disorders (1, 2). Experimental evidence gained from work on laboratory animals supports a delineation between the development of cellular immunity on the one hand and antibody synthesis on the other, and it is with the former that the thymus is most directly concerned (3). It is not surprising, therefore, that maldevelopment of the thymus is a central feature of diseases showing deficient cellular immunity. This paper is primarily concerned with the mechanisms by which some of these thymic anomalies may be produced.

The introduction of cell-marker techniques allows an analysis of thymic development which was previously impossible. Thus, a recent study in this laboratory has shown that stem cells migrate from the blood into the primitive thymic anlage where they proliferate and differentiate into lymphoid cells (4). In the present experiments thymic rudiments have been removed at various stages of development and cultured in diffusion chambers placed on the chick chorioallantois. By separating thymic rudiments from their stem cell supply in this way, it is hoped not only to gain insight into the importance of the interaction between them to normal thymic development, but also to provide an experimental model which may simulate some of the thymic defects found in vivo.

Materials and Methods

Thymic rudiments were dissected from (*a*) chick embryos of 6–9 days' incubation and (*b*) mouse embryos (CBA.H × BALB.C) of 11 to 14 days' gestation using a stereomicroscope (magnification of 16) and fine cataract knives. Rudiments were removed cleanly together with a thin rim of surrounding connective tissue. In chick embryos, primordia were removed separately from the right and left sides of each embryo: one side was fixed for histology; the other side was cultured. In mouse embryos, rudiments from some members of each litter were fixed immediately while rudiments from the rest of the litter were cultured. Groups of 10–15 rudiments were cultured at each stage. Gestation stage of mouse embryos was determined by noting the appearance of vaginal plugs (counted as day 0).

Diffusion Chamber Technique.—The method used was one modified from that described by Bell (5, 6) and subsequently used for the culture of human leukocytes (7). The advantages

of this technique are twofold; first the host embryo does not mount an immune reaction against donor tissue, and second, it provides a highly nutritive environment in which the full developmental potentiality of the isolated rudiments may be expressed.

Chambers were constructed by cementing Millipore nylon-reinforced (type TW) filter discs to one side of Plexiglas diffusion chamber rings (14 mm diameter) with Millipore MF cement. Thymic rudiments were carefully placed on the floor of each chamber which was left open. No fluid media was added to the chambers since they fill with nutritive fluid from the chorioallantois.

The chorioallantoic membranes of eggs of 7 or 8 days' incubation were dropped from

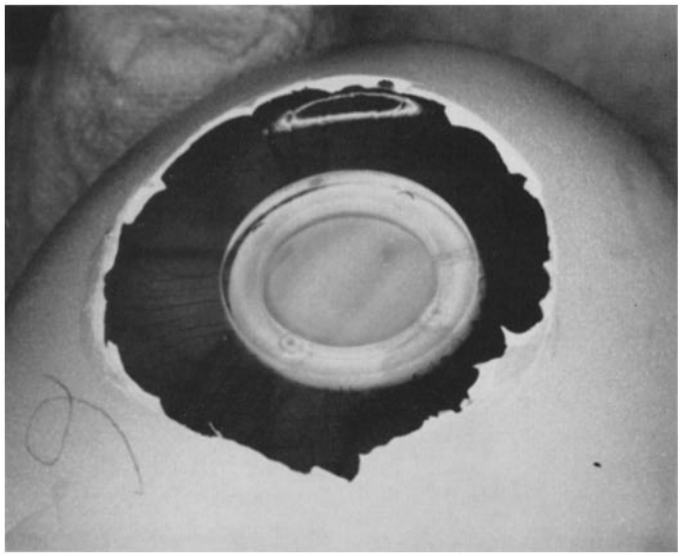

FIG. 1. Diffusion chamber embedded in chorioallantois. The circular cover slip sealing the hole in the shell has been removed and the hole enlarged to expose the chamber.

the shell membranes using a technique described by Hancox (8). Each diffusion chamber was carefully placed onto the chorioallantois through a hole cut in the shell (Fig. 1). Finally, the holes were sealed by waxing around a circular cover slip and the eggs were incubated in a horizontal position with the chambers lying on the "collapsed" chorioallantois.

Histological Methods.—At daily intervals up to 6 days after explantation thymic rudiments were removed from chambers and placed in Bouin's fixative. Both these rudiments and those fixed as controls were embedded in polyester wax and sectioned at 7 μ. Sections were stained in Giemsa, dehydrated in graded alcohols and mounted in De Pe X (G. Gurr Ltd., London, England).

RESULTS

Chick Thymic Rudiments.—The suitability of the diffusion chamber method for the culture of thymic primordia is shown by the excellent development and growth of rudiments removed from older embryos. At 9 days' incubation the chick thymus contains considerable numbers of cells which are characterized

by strongly basophilic cytoplasm and prominent nucleoli (Fig. 2). Evidence has been presented that these cells are the precursors of thymus lymphocytes (4). After 3 days' culture, these rudiments show considerable lymphoid development (Fig. 3) and this is maintained during the rest of the period under study. 8-day chick rudiments show a degree of lymphoid development which is only slightly less than that of 9-day rudiments.

The 7 day chick thymus is largely composed of pale-staining epithelial cells, although a few basophilic cells can be found within it (Fig. 4). These rudiments, even after 5 days' culture, contain only scattered lymphoid cells (Fig. 5) and they increase very little in size.

Histological examination of the 6 day thymic rudiment suggests that it is composed entirely of epithelial cells (Fig. 6); no basophilic cells have been found within it. The rudiment shows no lymphoid development in culture; indeed neither its histological nor gross appearance changes during a 6 day period (Fig. 7).

Chorioallantoic Grafting of 6-Day Chick Rudiments.—Since 6-day chick thymic rudiments fail to develop in diffusion chambers, a further experiment was carried out in which they were grafted directly on to the chorioallantois of 9-day chick embryos. In this site they rapidly become vascularized and after a period of 6 days have grown considerably and become fully lymphoid (Fig. 8). Their development and differentiation is comparable to that of the thymus *in situ* (Fig. 9).

Mouse Thymic Rudiments.—Both 14- and 13-day mouse thymic rudiments contain large numbers of basophilic cells (Fig. 10) and they undergo excellent lymphoid development and growth in diffusion chambers (Fig. 11). Basophilic cells are also found in 12-day rudiments (Fig. 12), which become lymphoid in culture (Fig. 13) but not to the extent shown by 13- or 14-day rudiments.

11-day mouse thymic rudiments contain very few basophilic cells, although they can be found in the mesenchyme around the primordium (Fig. 14). The thymus at this stage undergoes little further development when cultured in diffusion chambers and retains its pouch-like form (Fig. 15). After 6 days' culture a few lymphoid cells can be found within it.

Attempts were made to isolate thymic primordia from 10-day mouse embryos. However, they could not be dissected out cleanly and so blocks of tissue containing thymic primordia were cultured. On histological examination after a 7 day culture period no lymphoid tissue could be found.

DISCUSSION

These results show that the degree to which thymic rudiments will develop in diffusion chambers is critically dependent upon the age of the embryo from which they are removed. Thus, little or no development occurs in rudiments from early embryos, whereas excellent development takes place in rudiments

from older ones. Since in the chick at least, stem cells migrate from the blood into the thymic anlage (4), the most probable explanation for this result is that the potential development of a rudiment is governed by the number of migrant stem cells it has received before removal. The stem cells, once they have entered the rudiment apparently have only a limited capacity for proliferation. Their inability to self-replicate within the thymus limits its development once its supply of cells has been cut off.

6-day chick thymic rudiments fail both to become lymphoid or to increase in size during a 6 day period in diffusion chambers. This suggests that at this stage the chick thymus has not, as yet, received stem cells from the blood and hence cannot develop further in culture. If a stem cell supply is made available, as when the rudiment is grafted to the chorioallantois, it is able to become fully lymphoid. Under these circumstances the lymphoid cells within the graft will be of host origin. It has already been shown by means of chromosome marker methods that chick thymic grafts made to the chorioallantois become populated by host cells (4).

The relatively poor development of 7-day chick rudiments in chambers is presumably due to the fact that they contain only a few stem cells. By contrast, 9-day rudiments have sufficient stem cells to develop in a normal manner at least for a 6-day period. There is a marked correlation between the capacity of rudiments to become lymphoid and the number of basophilic cells they contain. This supports previous evidence (4) that these cells are migrant stem cells.

No lymphoid development was found when blocks of tissue containing the 10 day mouse thymic primordium were cultured and so it seems likely that, as in the 6 day chick thymus, there are no stem cells in the rudiment at this stage. 11-day mouse thymic rudiments undergo very little lymphoid development in diffusion chambers which suggests that they contain only a few stem cells.

There are a greater number of stem cells in the 12 day mouse rudiment since it shows good lymphoid development in culture. This confirms the observations of Auerbach (9). However, the results of this study and a previous one (4) do not support his contention that these stem cells are derived from the epithelial cells of the organ anlage. On the contrary, our evidence suggests that the 12 day mouse thymus can become lymphoid in culture because it contains stem cells derived from the blood stream. The 14 day mouse rudiment develops even further in culture because it contains a greater number of stem cells derived from this source. It seems likely that, as in the chick, the basophilic cells found within the developing mouse thymus are migrant stem cells.

No attempt was made to graft early mouse thymic primordia to the chick chorioallantois so as to restore a supply of stem cells, since it has been shown previously that avian stem cells will not proliferate within a mouse rudiment

(4). However, our recent unpublished results show that it is possible to obtain full lymphoid development of very early mouse thymic primordia by grafting them under the kidney capsule of syngeneic hosts where they reach weights of up to 30 mg after 28 days. Use of the T6 chromosome marker has shown that these grafts become populated by host cells.

The experiments reported here emphasize the importance of the interaction between stem cell and organ anlage to normal thymic development. Clearly, some of the developmental anomalies found clinically may be due to a failure in one or both of these components. A condition of complete thymic agenesis has been reported (10, 11) in which the third and fourth pharyngeal pouches fail to develop. As a result no epithelial thymic anlage is formed and since it is this rather than the stem cell which is deficient, the most effective treatment for the condition should be the implantation of a thymic graft.

In other deficiency diseases the thymus develops as an epithelial rudiment but remains alymphoid. In one of these (reticular dysgenesia) other blood leukocytes also fail to develop (12, 13). It seems reasonable to conclude that both defects may be due to the absence of a multipotential stem cell. Evidence has already been presented that during embryogenesis circulating stem cells have a number of developmental potentialities (14, 15). The basis of the thymic defect in this disease may therefore be comparable to that shown by the early thymic rudiment explanted in diffusion chambers, where the absence of a stem cell supply leads to a total failure of lymphoid development. Just as restoration of this supply produces full development of the embryonic thymus, so the alymphoplasia found in this disease may perhaps be reversed by the injection of normal fetal hemopoietic cells.

The situation is likely to be more complex in other conditions of thymic failure whether or not they are associated with agamma globulinemia. Lymphocytes may be present in the thymus in either situation (16, 17) and there is no evidence of a general stem cell deficiency as in reticular dysgenesia. Although the thymic anlage is formed, it is possible that it is functionally defective and fails to influence the differentiation of inflowing stem cells in a lymphoid direction. A basis for the development of a defective thymic anlage has been suggested by Peterson et al. (18) in ataxia-telangiectasia. In this disease there is a generalized mesenchymal defect which might result in the maldevelopment of the thymic rudiment, since it has been shown that the presence of mesenchyme is necessary for the formation of the epithelial anlage (19).

It seems likely, therefore, that many of the thymic anomalies found in immunological deficiency diseases can be accounted for by a developmental failure in the stem cell/organ rudiment interaction. Further study of the nature of this interaction should be important not only to an understanding of these diseases but also to the processes of immunogenesis.

SUMMARY

Thymic rudiments from chick and mouse embryos have been cultured in diffusion chambers on the chick chorioallantois. In this situation their supply of blood-borne stem cells is cut off. Although rudiments from older embryos become fully lymphoid under these circumstances, primordia from early embryos fail to develop any lymphoid cells. Early chick rudiments will however develop completely if they are grafted directly to the chorioallantois where they receive a vascular supply.

It is concluded that stem cells first enter the chick thymic anlage at between 6 and 7 days' incubation and the mouse thymic rudiment between 10 and 11 days' gestation. During the following few days of development there is a rapid inflow of stem cells to the rudiments. Since it is likely that stem cells, once they have entered the thymic primordium, are capable of only limited proliferation, it must be concluded that an inflow of stem cells continues once full lymphopoiesis has begun, although perhaps at a reduced rate.

Finally, the importance of the interaction between stem cell and organ rudiment to normal thymic development is discussed in relation to the pathogenesis of thymic anomalies.

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FIG. 2. Thymic rudiment of 9 day embryo. Note the presence of cells with darkly staining (basophilic) cytoplasm within rudiment. Giemsa stain. × 2250.

FIG. 3. 9 day chick thymic rudiment cultured for 3 days in a diffusion chamber (giving a total developmental age of 12 days). There are numerous lymphoid cells, characterized by speckled chromatin, within the rudiment. Giemsa stain. × 2250.

FIG. 4. Section of 7 day chick thymic rudiment. At this stage it is largely composed of pale-staining epithelial cells but a few cells with darkly staining cytoplasm can be found within it. One of these is arrowed. Giemsa stain. × 2250.

FIG. 5. 7 day chick thymic rudiment cultured for 5 days giving a total developmental age equivalent to that of the cultured 9 day rudiment in Fig. 3. However, the degree of lymphoid development is considerably less than that shown by the 9 day rudiment. Giemsa stain. × 2250.

FIG. 6. Thymic rudiment of 6 day chick embryo. The rudiment is entirely composed of epithelial cells and no basophilic cells can be found within it. Giemsa stain. × 2250.

FIG. 7. 6 day chick thymic rudiment cultured for 6 days. Although this rudiment should now be at the equivalent developmental stage to the rudiments in Figs. 3 and 5, no lymphoid development has occurred within it and it remains entirely epithelial. Giemsa stain. × 2250.

FIG. 8. 6 day chick thymic rudiment grafted to the chorioallantois for 6 days. With the restoration of a blood supply the rudiment has become fully lymphoid. This field shows only a small portion of a large piece of thymic tissue. Its development compares favorably with that of the normal thymus *in situ* (see Fig. 9). Giemsa stain. × 2250.

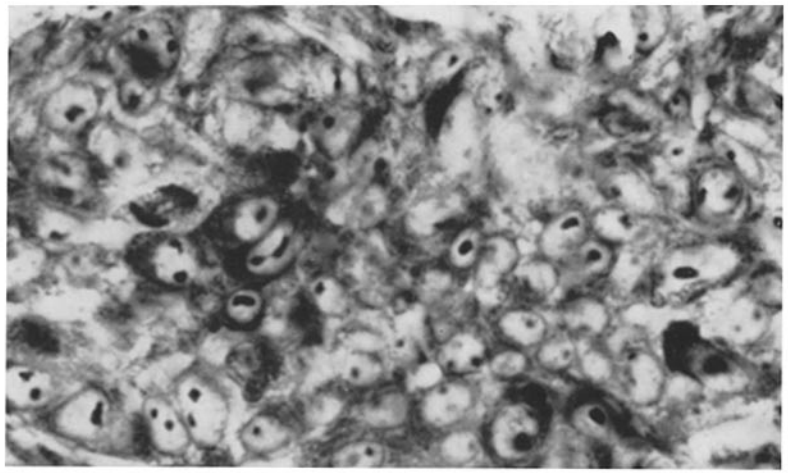
FIG. 9. Section of normal 12 day chick thymus. Giemsa stain. × 2250.

FIG. 10. 14 day mouse thymic rudiment. There are a large number of darkly staining (basophilic) cells within the rudiment. Giemsa stain. × 2250.

FIG. 11. 14 day mouse rudiment cultured for 3 days (total developmental time 17 days). The rudiment contains numerous lymphoid cells after this period. Giemsa stain. × 2250.

FIG. 12. Thymic rudiment of 12 day mouse embryo. The rudiment at this stage contains fewer basophilic cells than at 14 days. Giemsa stain. × 2250.

FIG. 13. 12 day mouse thymic rudiment cultured for 5 days. There is good lymphoid development within the rudiment. Giemsa stain. × 2250.



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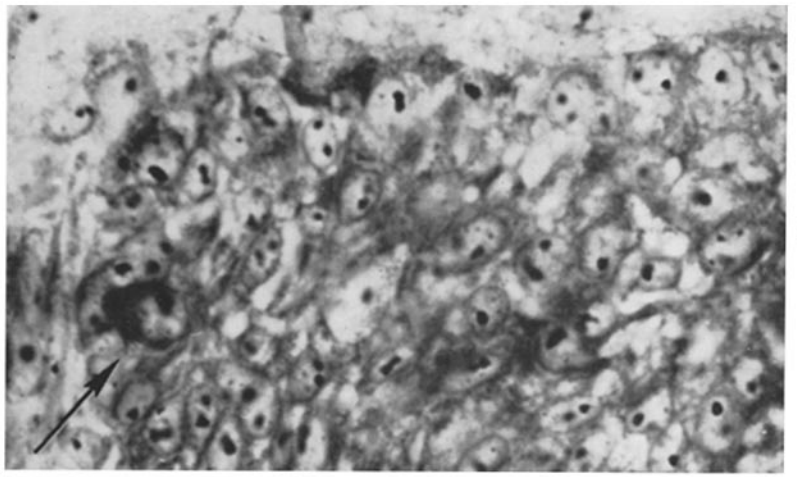

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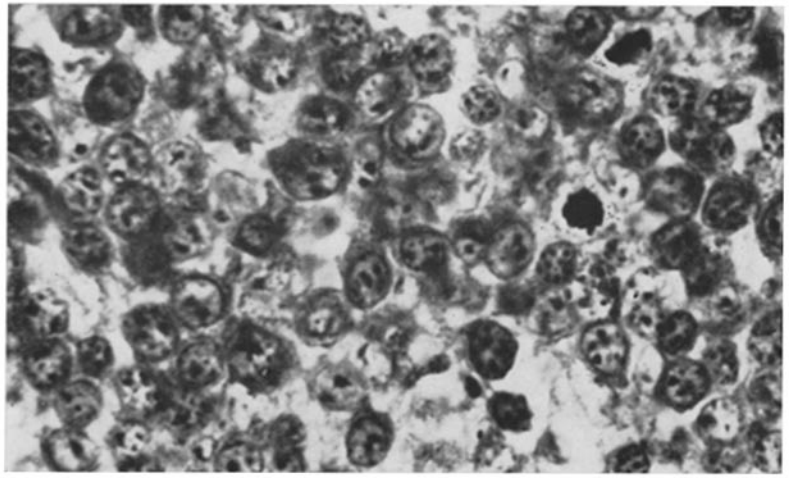

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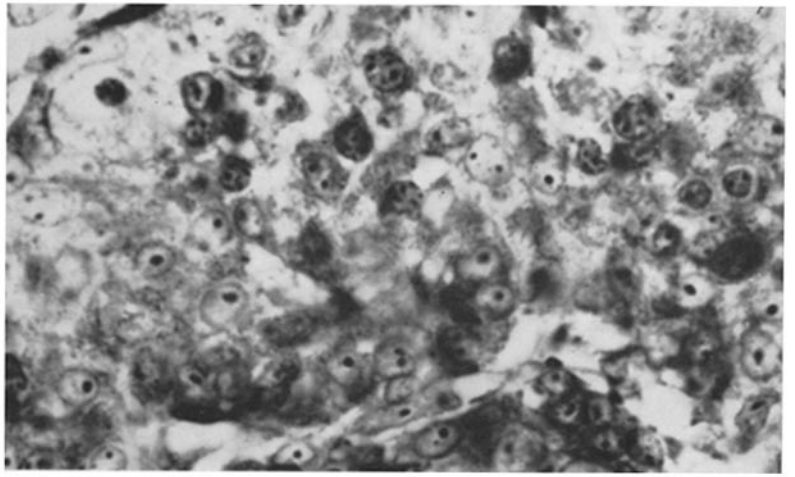

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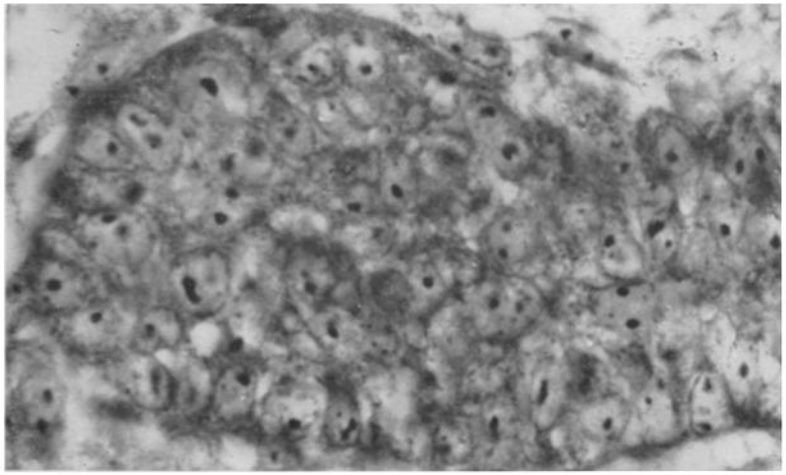

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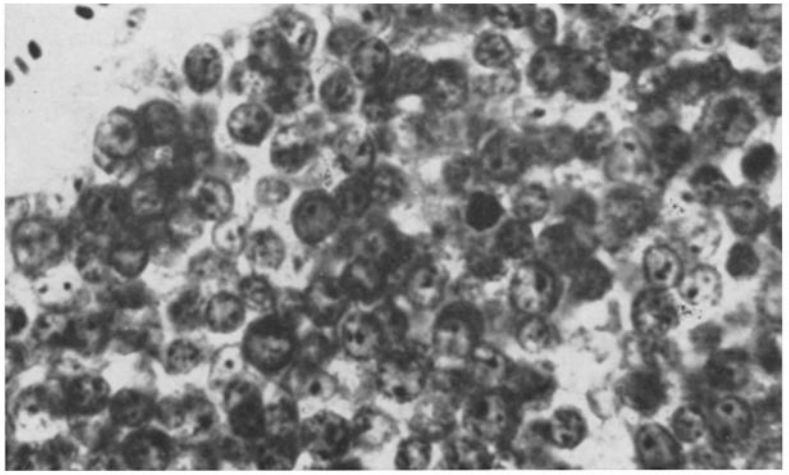

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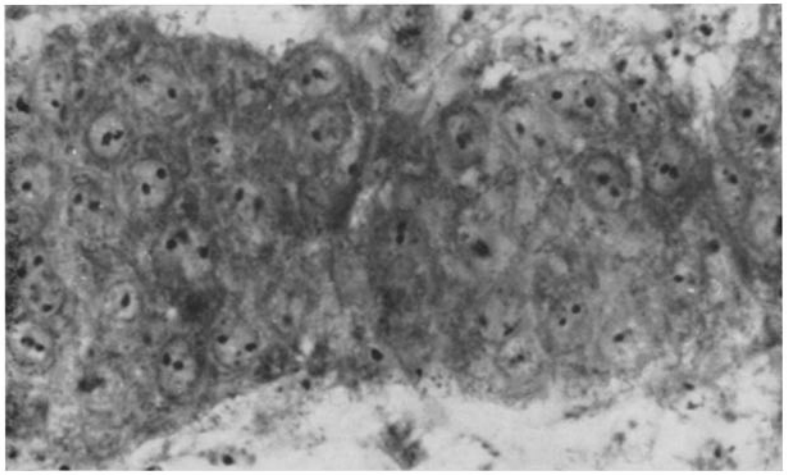

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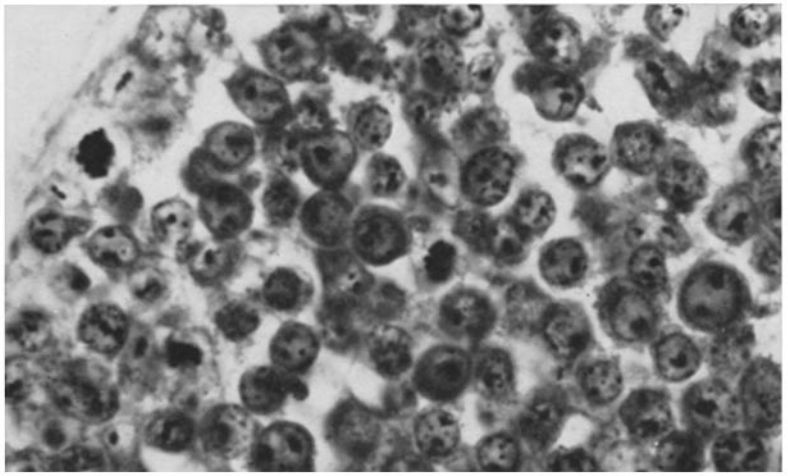

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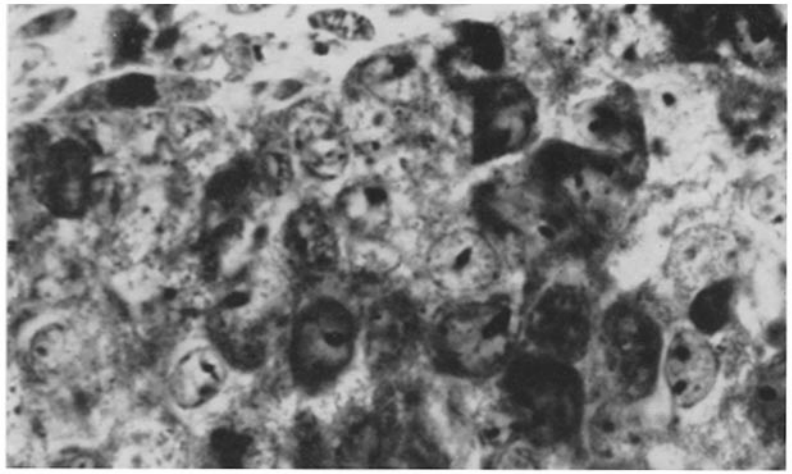

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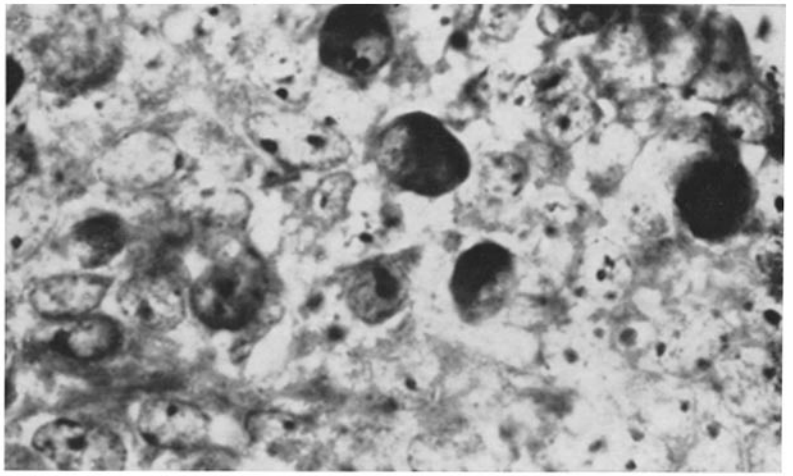

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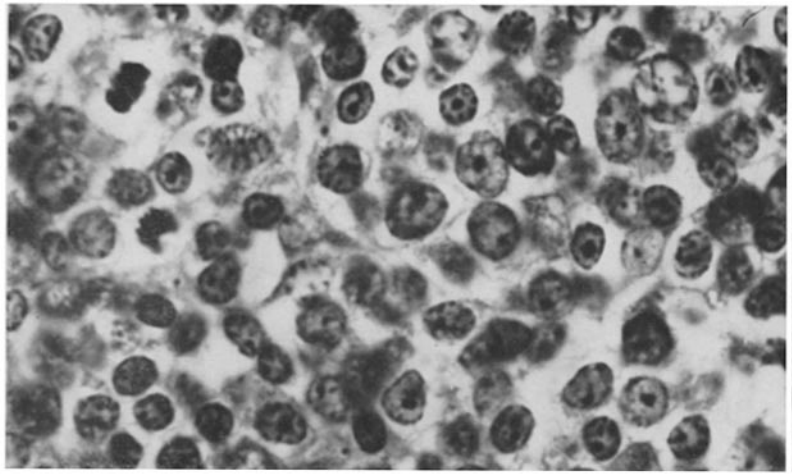

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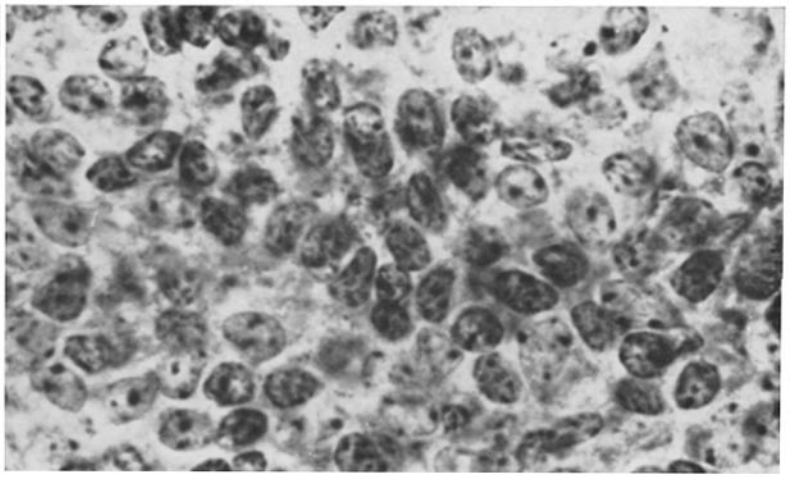

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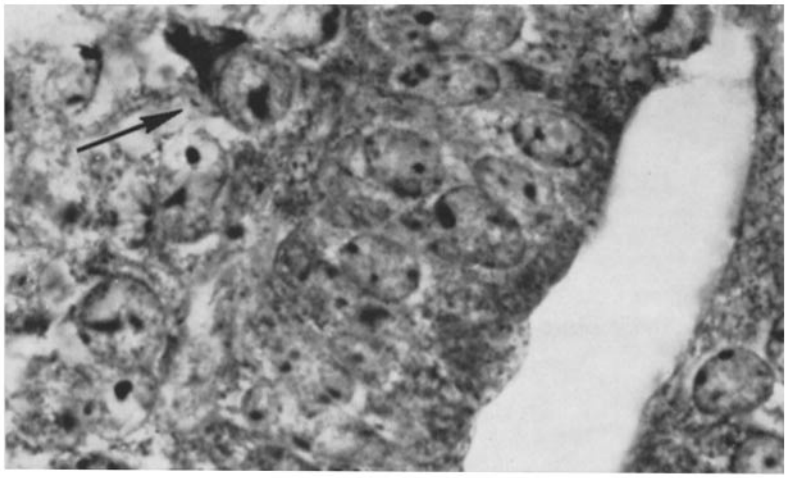

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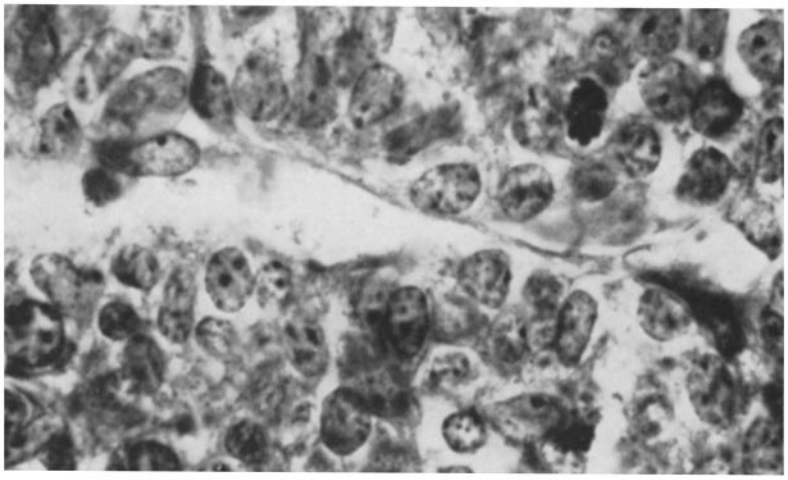

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FIG. 14. Thymic primordium of 11 day mouse embryo. A basophilic cell (arrowed) can be seen immediately adjacent to the rudiment. Giemsa stain. × 2250.

FIG. 15. 11 day mouse rudiment cultured for 6 days, giving a developmental age which should be equivalent to that of the rudiments in Figs. 11 and 13. However, it contains few lymphoid cells and has retained a pouch-like form. Giemsa stain. × 2250.