

IN VITRO STUDIES ON RADIATION LYMPHOID RECOVERY OF
MOUSE SPLEEN*· †

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PLATES 4 TO 7

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Studies on the role of the thymus in the adult have demonstrated its essential participation in lymphoid (2-4) and immunological (5, 6) recovery following total body x-irradiation. Subsequent investigations have suggested that the thymus acts indirectly (4, 7), probably by a humoral factor (8) as previously shown for lymphoid development during early postnatal life (9, 10). Although various attempts to demonstrate an active lymphoid stimulatory factor by using extracts have been reported (11-13) very little is known concerning the precise nature of these extracts. Furthermore, it has not yet been established what the mechanism of the lymphoid-stimulating effect is, whether it operates directly on the lymphoid-depleted tissue, whether stem cells are stimulated first to immigrate to the depleted sites, or whether it acts indirectly.

In vitro analytical studies on ontogenic lymphoid development have indicated that the thymus had no stimulatory effect on lymphopoiesis in the embryonic prelymphoid spleen (14), and the suggestion was made that at this stage the spleen sequesters lymphoid cells (15, 16) which originate in the thymus yet probably circulate through the bone marrow (17).

In view of the similarity between experimental results on lymphopoiesis in neonatal and adult-irradiated mice subjected to thymectomy, one might wonder whether the lymphoid regeneration process operates by the same mechanism as embryonic lymphoid development. In this context, the question was raised whether lymphoid cell migration and repopulation (18, 19) play an essential role in regeneration.

The present study was therefore undertaken to follow lymphoid regeneration of irradiated adult mouse spleen *in vitro*. The organ culture method (20, 21)

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was employed to permit critical analysis of the mutual interactions between individual tissues.

Experiments were designed to test: (a) whether the thymus has any direct stimulatory effect on lymphoid regeneration of the spleen; (b) whether cell migration to the spleen is essential for lymphoid regeneration; and (c) what function might be played by bone marrow or lymph nodes in lymphoid regeneration.

Materials and Methods

Mice.—C3H/He/Au mice were used as donors in all the experiments. Spleen and thymus were taken from 2- to 3-month-old mice, and bone marrow from 3- to 6-week-old mice.

X-Irradiation.—was performed at 140 kvp, 5 ma, 0.5 mm Cu, 1.0 mm Al, at a dose rate of 20 roentgen/minute. Mice were exposed to 550 r total body irradiation.

Culture Method.—The millipore filter well technique was used as previously described (20-22). Tissues were combined and kept for 1 day on top of the millipore filter to let them fuse; then they were transferred into the well. Eagle's basal medium was used, supplemented with 10 per cent horse serum (Difco Laboratories, Inc., Detroit No. 0569), 5 per cent chick embryo extract and antibiotics (penicillin, streptomycin, mycostatin, and erythromycin 50 units/ml each).

Incubation was carried out at 37.5°C in a water-saturated atmosphere with a gas phase of 95 per cent oxygen and 5 per cent CO₂.

Histology.—Tissues were fixed in Zenker's, sectioned at 5 to 7 μ , and stained with hematoxylin and eosin.

Preparation of Tissues.—Spleens were removed 24 hours after irradiation, and dissected into fragments (0.3 mm thickness and 0.5 to 0.7 mm diameter). Thymus fragments were prepared in a similar way. Bone marrow was gently removed from femurs, intact material from the bone was used for each culture.

EXPERIMENTAL

Thymus Interaction with Isolated Spleen Explants.—Previous studies on lymphoid recovery after radiation had demonstrated that spleens were depleted of lymphoid elements if thymectomy preceded exposure (2-4), and no subsequent regeneration took place unless a thymus was regrafted (4, 7, 8). However, a recent report on a culturing method of adult normal spleens indicated reformation of follicles following lymphoid depletion *in vitro* (23).

The first series of experiments was therefore attempted to test whether singly isolated spleen explants from irradiated mice are capable of spontaneous regeneration *in vitro*.

A total of 72 cultures from 36 donors were employed for this study. Tissues were fixed for histology on the day of culturing and after 1, 2, 3, 4, 6, 7, 8, 9, 10, 12, 13, 14, 15, 20, and 42 days.

Daily inspection of cultures revealed a gradual damage in architecture. Red blood cells were found at the edge of the explants, and the color of tissues turned to yellowish-brown. White pulp areas lost their distinct structures, and by the end of the first week spleen fragments appeared as homogeneous transparent

tissues, with no obvious organization of white and red pulp. Histological sections examined during the first week of culture revealed lymphoid cells scattered at various sites in the tissue with no spatial connection to follicles (Fig. 1 *a*). Occasionally follicle remains were detected, containing some lymphocytes, usually pyknotic (Fig. 1 *b*). No major changes were observed at later periods. The conclusion was therefore made, that under these experimental conditions no lymphoid regeneration could be detected in irradiated spleens.

The next experiments were designed to test whether lymphopoiesis might be stimulated in the presence of thymus. Thymuses were isolated from normal adult C3H mice. Spleen and thymus fragments were combined in 11 cultures. The same number of controls were studied in parallel, in which singly isolated spleens and thymuses were compared. Cultures were checked daily as before, for a period of 6 weeks.

Spleen tissues in combination cultures underwent the same changes as singly isolated ones, with a distinct border visible at the site of connection with the thymus (Fig. 2). No lymphoid regeneration was observed, and even when lymphoid follicles were placed in close connection with thymus tissue they were devoid of lymphocytes during the period of culturing. The thymus, on the other hand, was lymphoid, but no lymphocytes were found in the spleen at the border area between the tissues.

It therefore appears as if no direct migration of lymphocytes took place from thymus to spleen, nor was there any obvious stimulatory effect of thymus on spleen lymphoid regeneration.

A second series of experiments was undertaken to test whether thymus from irradiated mice would stimulate lymphopoiesis, the assumption being that thymuses undergoing regeneration might be more effective. Thymuses were isolated at two different time intervals, 3 hours and 1 day after irradiation. Nine spleen fragments from 6 donors were combined with thymus taken 3 hours after irradiation and 19 spleen fragments from 13 donors were studied while combined with thymus removed 1 day after exposure. Equivalent number of controls, in which thymuses and spleens were singly isolated were cultured in parallel for comparison. In all of the cultures where spleens were placed together with thymus, regardless of the pretreatment to thymus no lymphoid regeneration of spleen was detected (Fig. 3).

Interaction of Irradiated Spleen with Bone Marrow.—The previous experiments indicated that in this culture system no stimulatory effect of thymus on recovery of irradiated spleen explants could be observed. Similar observations have been reported on interaction between embryonic prelymphoid spleen with thymus (14). On the other hand, studies on embryonic development of spleen indicated the role of cell migration (14–16), and recent evidence pointed to the participation of bone marrow in cell donation (17). In view of these considerations, and the additional fact that bone marrow is capable of conferring protec-

tion from lethal doses of irradiation (24), experiments were planned to test the possible interaction of irradiated spleen with adult bone marrow *in vitro*.

Bone marrow was dissected from 3- to 4-week-old syngeneic mice. Intact material from each femur was combined with one spleen fragment. A total of 24 cultures were studied, with the same number of singly isolated spleens and bone marrows for controls.

Within the first week of culture, singly isolated spleens underwent lymphoid depletion as previously described, yet while combined with bone marrow, the spleens had lymphoid areas (Fig. 4), consisting of small and medium sized lymphocytes. Lymphocytes were also detected in the bone marrow region (Fig. 4) of such combination cultures, although they were rare in the singly isolated bone marrow explants (Figs. 5 and 7 *a*).

It seems likely that lymphocytes in these cultures originated in the bone marrow. To test whether spleen might interact with other tissues in a similar way, or whether this synergistic interaction is unique to spleen and bone marrow, further experiments were carried out in which mesenteric lymph nodes from untreated adult mice were combined with irradiated spleens (7 cultures). In such cultures, however, no interaction occurred, spleen and lymph nodes in combination showing no difference from the singly isolated tissues. It appears, therefore, as if interaction observed between bone marrow and spleen is specific to these tissues.

In view of the demonstrated effect of thymus on lymphopoiesis in thymectomized mice irradiated at lethal doses and treated with bone marrow (7, 8) the following experiments were designed to test whether thymus has any effect on spleen-bone-marrow combination cultures.

Irradiated spleen fragments were combined with bone marrow as before, but thymus from either normal (8 cases) or irradiated (14 cases) donors was also added in a manner which allowed contact with both bone marrow and spleen tissues. In all of these cultures bone marrow and spleen were obviously lymphoid on the 6th to 8th day of culture (Figs. 6 and 7 *b*). Furthermore where spleen lymphoid follicles were placed in contact with thymus and bone marrow active lymphopoiesis occurred. However, in control cultures where irradiated thymus was combined with bone marrow only (23 cases) no lymphopoiesis was noticed in the bone marrow; the marrow consisted mainly of granulocytes at various stages of differentiation, with frequent mitotic figures (Fig. 7 *c*).

These experiments therefore point to a mutual interaction between irradiated spleen and bone marrow, which lead to appearance of lymphoid cells; and which is enhanced by the thymus.

DISCUSSION

The present study demonstrates a synergistic interaction between irradiated spleen and normal bone marrow. It appears as if bone marrow lymphoid cells

are specifically stimulated in the presence of irradiated spleen and that in return the spleen gains such cells. Singly isolated bone marrow or spleen are not lymphoid under the same experimental conditions. That this is probably a result of specific interaction between the tissues is indicated by the observation that irradiated thymus did not confer such an effect on bone marrow, nor did the spleen gain lymphocytes from normal thymus or lymph node. On the other hand, thymus did not stimulate lymphoid regeneration in spleen while the two tissues were combined in culture; unless bone marrow was also present. These results are entirely in line with experimental evidence from the development of the embryonic system (1, 17), where similar interactions have been shown.

The present study utilized an artificial situation of a close contact between tissues, which is far from being the case *in vivo*. However, various reports have shown circulation of bone marrow cells to spleen (25-27) and thymus (28). Furthermore, spleen grafts were reported to gain lymphoid cell population from the host (29, 15, 17) similarly to thymus grafts (30). One is therefore drawn to postulate that inductive interactions occur *in vivo* by a cell to cell contact within the regenerating tissue (see reference 7). Such inductive interactions have been well documented for many embryonic systems (20) and were proposed as a possible control mechanism in the adult (31). This is in line with early investigations on leukemia development (32) in thymectomized and irradiated mice which developed leukemia upon regrafting with thymus. Such leukemias developed within the graft yet some of the leukemic cells were of host and some of donor origin, suggesting that host cells were induced within the thymus graft to become leukemic. Similar inductive interaction was shown in an *in vitro* system, where bone marrow induced lymphoid regeneration of thymus previously exposed to urethan (33). The present study extends such a possible mechanism to spleen and bone marrow suggesting that bone marrow cells are specifically stimulated within the irradiated spleen environment.

This study was not planned to establish the mechanism of lymphoid recovery within bone marrow subjected to x-irradiation. Whether this recovery is induced by thymus is an open question so far.

SUMMARY

In vitro studies, utilizing an organ culture method were reported on mutual interactions between irradiated spleen, normal bone marrow, and thymus. It has been shown; (a) that singly isolated spleen explants were incapable of lymphoid regeneration, (b) thymus had no stimulatory effect on spleen regeneration, (c) bone marrow interacted synergistically with spleen leading to appearance of lymphoid cells which were not detected in singly isolated bone marrow or spleen, and (d) no stimulation of lymphopoiesis in bone marrow was conferred by thymus in the absence of spleen.

The results are discussed in terms of possible mechanisms involved in lymphoid radiation recovery *in vivo*.

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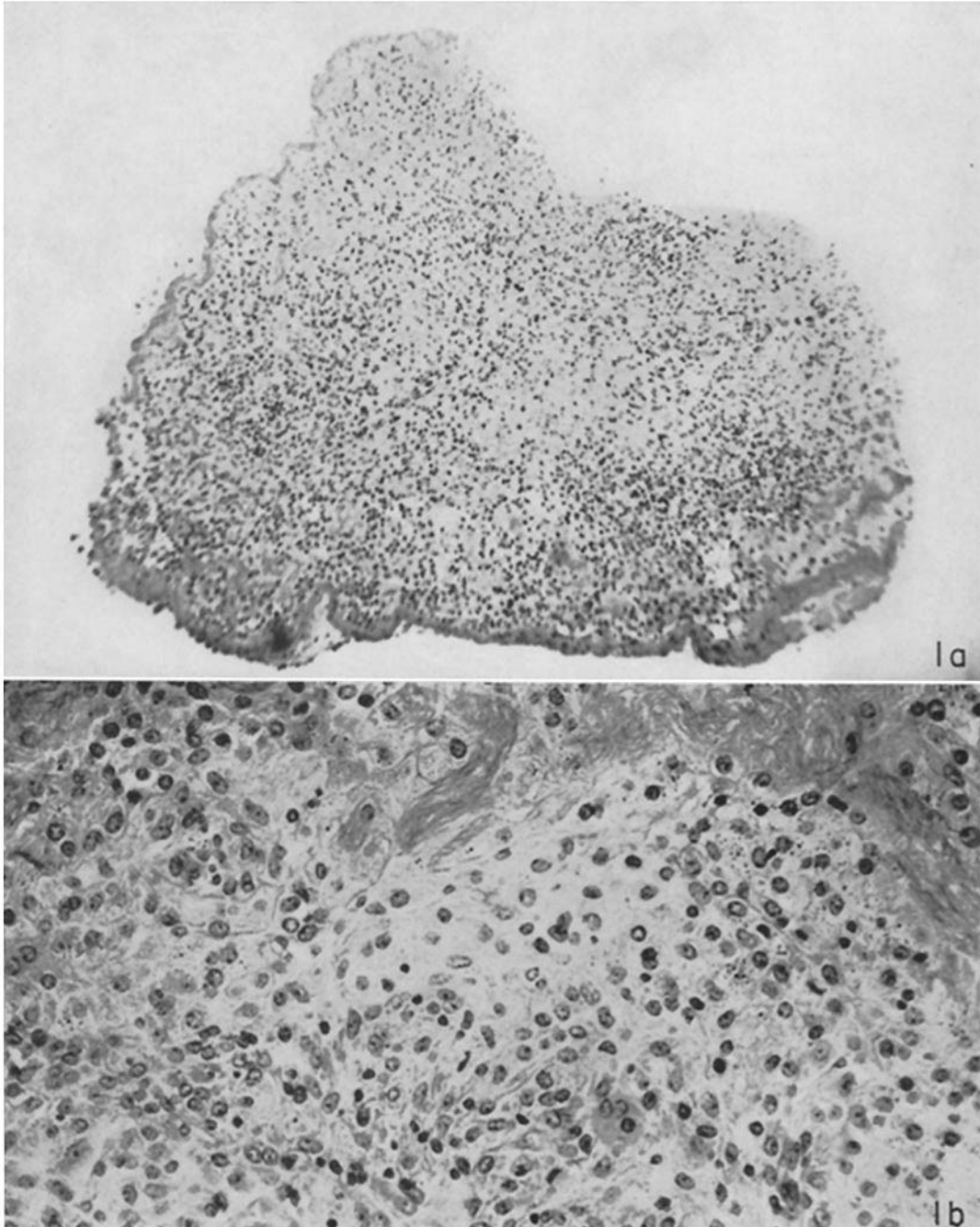
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EXPLANATION OF PLATES

PLATE 4

FIGS. 1 *a* and 1 *b*. Explants of irradiated spleen. Fig. 1 *a*, 4 days in culture, $\times 177$.
Fig. 1 *b*, 6 days in culture, $\times 568$.

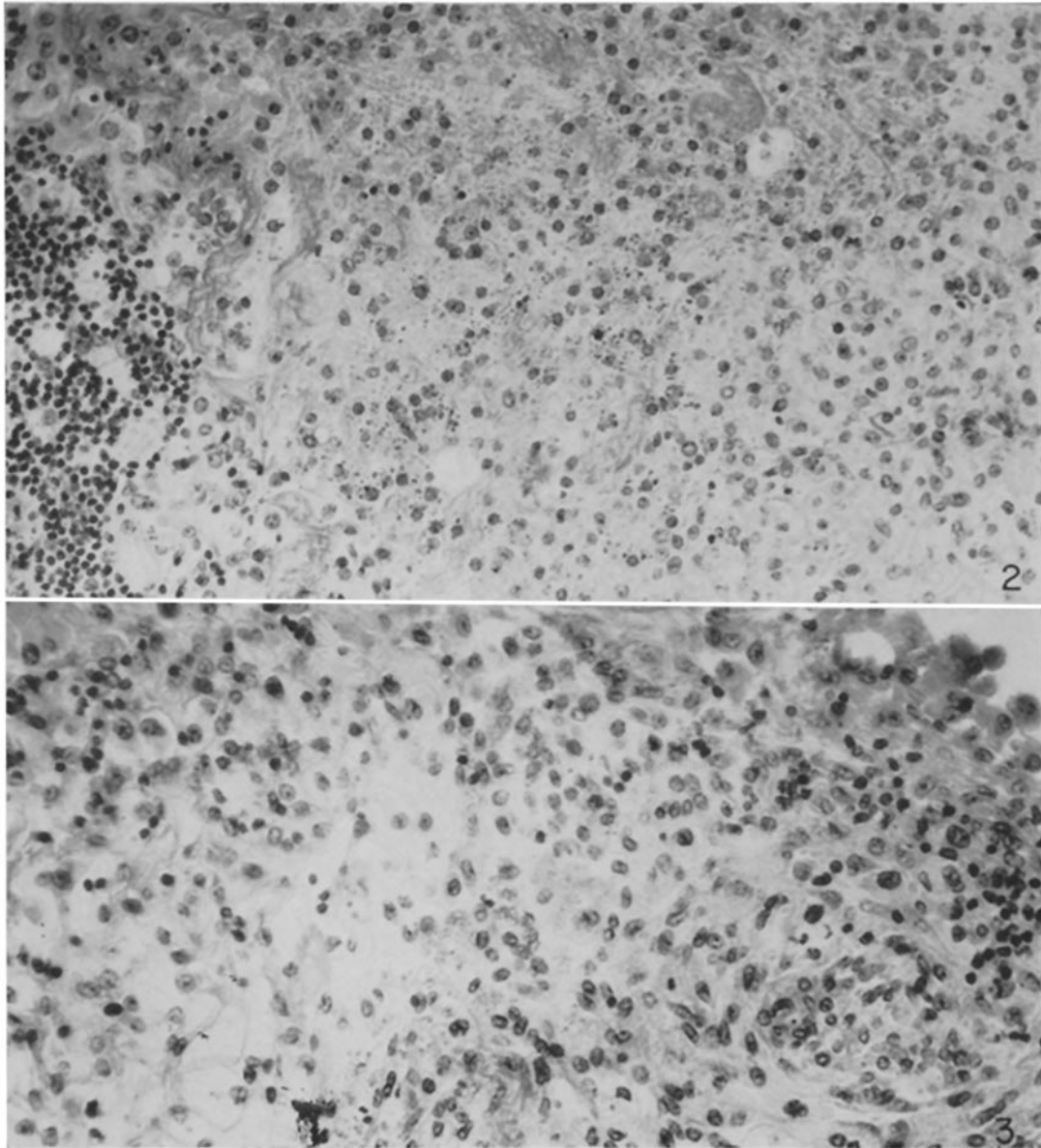


(Globerson: *In vitro* lymphoid regeneration)

PLATE 5

FIG. 2. Tissue combination of irradiated spleen (at right) and thymus from an untreated mouse (at left). 6 days in culture, \times 413.

FIG. 3. Tissue combination of irradiated spleen (at right) and thymus from an irradiated mouse, removed 1 day following exposure (at left). A lymphoid follicle is observed at the spleen area, containing some lymphocytes with pycnotic nuclei. 7 days in culture, \times 558.



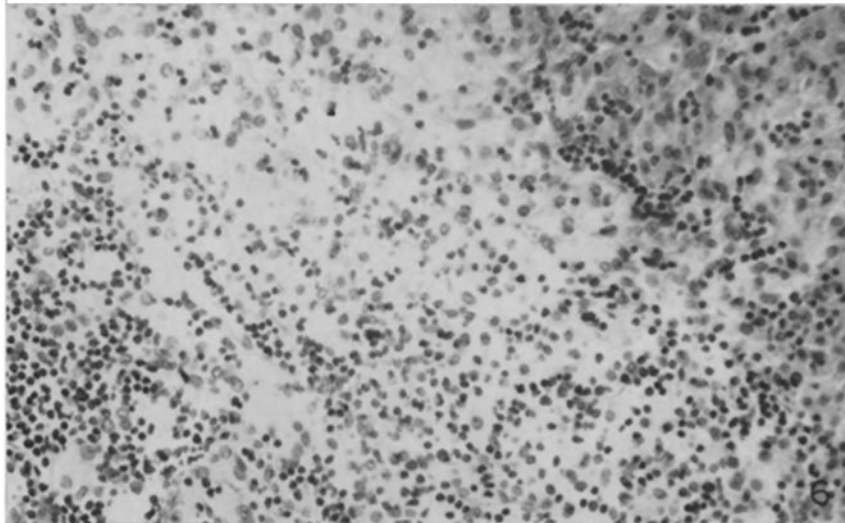
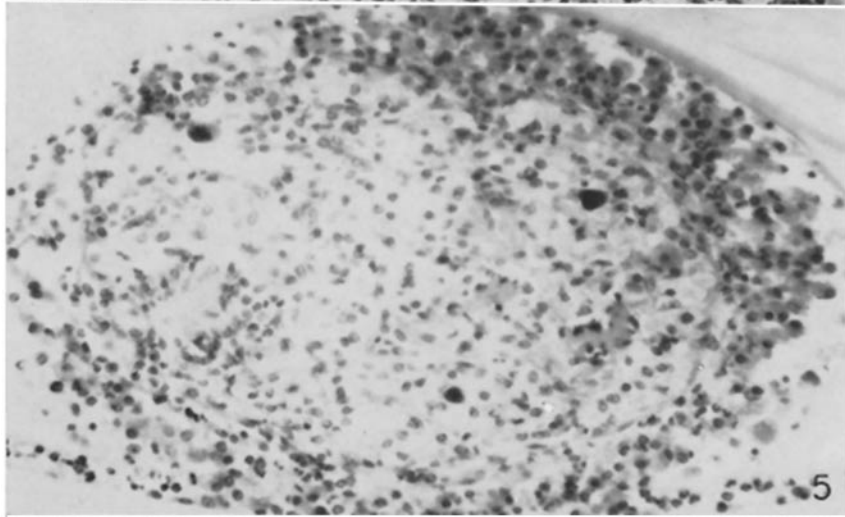
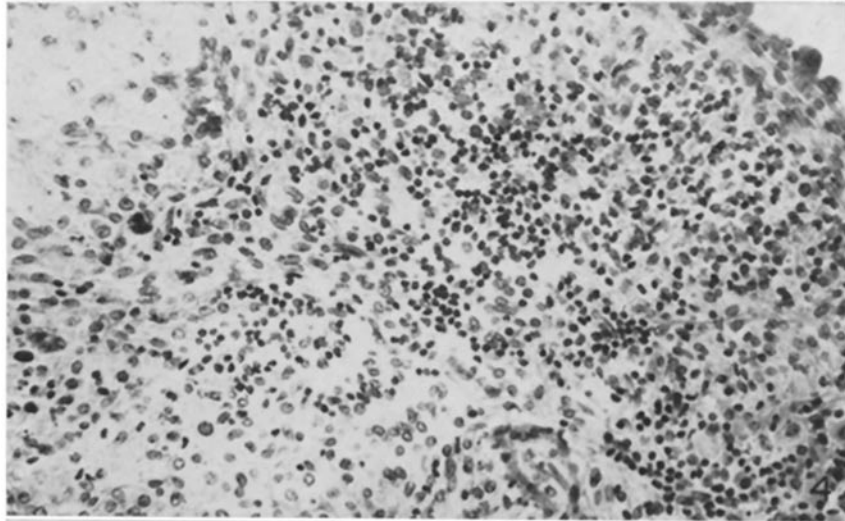
(Globerson: *In vitro* lymphoid regeneration)

PLATE 6

FIG. 4. Bone marrow region in a spleen-bone marrow culture. A part of the spleen tissue is observed at left, below, containing lymphocytes. 6 days in culture, $\times 366$.

FIG. 5. Bone marrow culture, singly isolated. The tissue is surrounded by macrophages and a few granulocytes. 6 days in culture, $\times 366$.

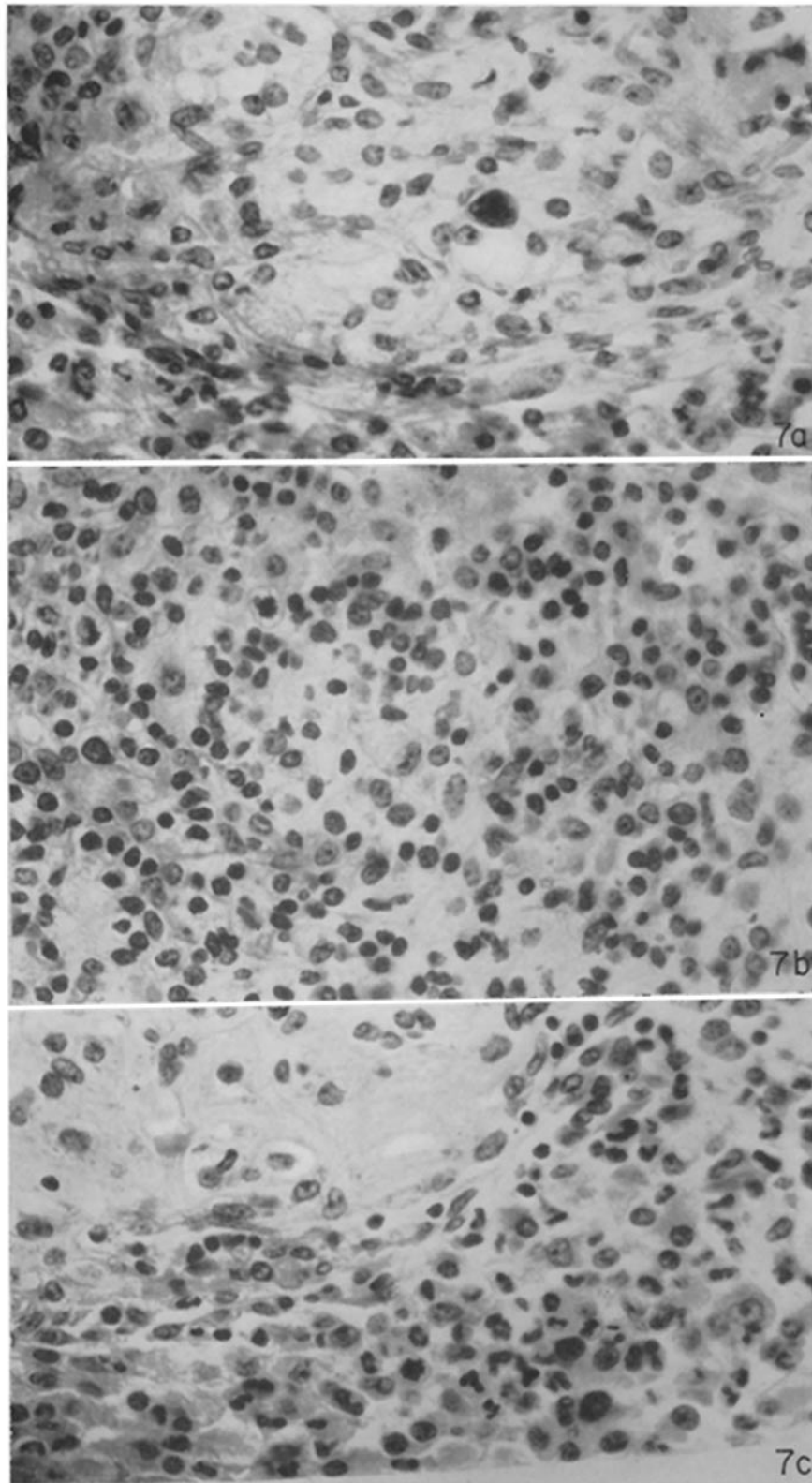
FIG. 6. Tissue combination of irradiated spleen (at upper right), normal thymus (at left) and bone marrow. 6 days in culture, $\times 366$.



(Globerson: *In vitro* lymphoid regeneration)

PLATE 7

FIGS. 7 *a* to 7 *c*. Bone marrow, 6 days in culture. Fig. 7 *a*, singly isolated; Fig. 7 *b*, in combination with irradiated spleen and thymus from an untreated mouse; and Fig. 7 *c*, in combination with thymus from irradiated mouse. Removed 3 hours after exposure, $\times 820$.



(Globerson: *In vitro* lymphoid regeneration)