Restoration of reactivity in old animals was achieved on account of activation of proliferation of the salivary gland cells (Table 1).

To assess the effect of the drugs on the lymphoid system, activation of which was demonstrated in the course of isoproterenol-induced hyperplasia of the salivary glands in mice and rats [2], an approach based on the isolation of activated lymphocytes, which are larger and have a lower specific gravity [12], was used. It will be clear from Table 1 that the number of these lymphocytes, isolated 4 h after injection of isoproterenol into old animals, was greatly increased in mice receiving dopamine, but reduced in mice receiving serotonin.

Excitation of serotoninergic structures thus inhibits, whereas excitation of dopaminergic structures stimulates (like its effect on immune reactions [3]) the response of mice to isoproterenol. Since the action of these substances was accompanied by a corresponding simultaneous decrease and increase in the number of active splenic lymphocytes which, as the writers have shown [2], are responsible for induction and for restriction of proliferation of salivary gland cells, it can be concluded that the action of serotonin and dopamine is mediated through its effect on the lymphoid system.

LITERATURE CITED

1. A. G. Babaeva, N. A. Kraskina, and L. D. Liozner, Byull. Eksp. Biol. Med., No. 2, 78 (1973).
2. L. V. Devoino and R. Yu. Il'ichenko, Monoaminergic Systems in the Regulation of Immune Reactions [in Russian], Novosibirsk (1983).
3. V. I. Dontsov, in: Abstracts of Proceedings of the 7th All-Union Conference on Regeneration and Cell Multiplication [in Russian], Moscow (1985), pp. 87-88.
4. R. V. Petrov and L. S. Seslavina, Zh. Mikrobiol., No. 11, 28 (1977).
5. R. C. Adelman, Exp. Geront., 6, 75 (1971).
6. T. Barka, Exp. Cell Res., 39, 355 (1965).
7. R. E. Callard, A. Basten, and K. Wasters, J. Immunol., 124, 52 (1980).
8. C. E. Finch, Brain Res., 52, 261 (1973).
9. J. J. Liu, M. Segre, and D. Segre, Cell. Immunol., 66, 372 (1982).
10. A. M. Preumont, P. van Gansen, and J. Brachet, Mech. Age Dev., 7, 25 (1978).
11. D. S. Robinson, Fed. Proc., 34, 103 (1975).
12. J. G. Salisbury, J. M. Graham, and C. A. Pasternak, Br. J. Cancer, 40, 307 (1978).
13. J. D. Thrasher and R. C. Greulich, J. Exp. Zool., 159, 39 (1965).

BONE FORMATION IN BONE MARROW ORGAN CULTURES

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Evidence that osteogenic precursor cells are present in bone marrow has been obtained in heterotopic transplantation experiments. Fragment of bone marrow, when transplanted in diffusion chambers, form bone tissue [2, 6], and if transplanted beneath the renal capsule, they form bone marrow organs in which bone, osteogenic cells, and colony-forming stromal mechanocytes of the medullary cavity are of donor origin [3, 5]. Osteogenic cells also are present in suspensions of bone marrow cells. If the latter are transplanted in diffusion chambers, bone and cartilage are formed [6, 9], and transplantation of 10^5 bone marrow cells beneath the renal capsule leads to the formation of a heterotopic bone marrow organ [4].

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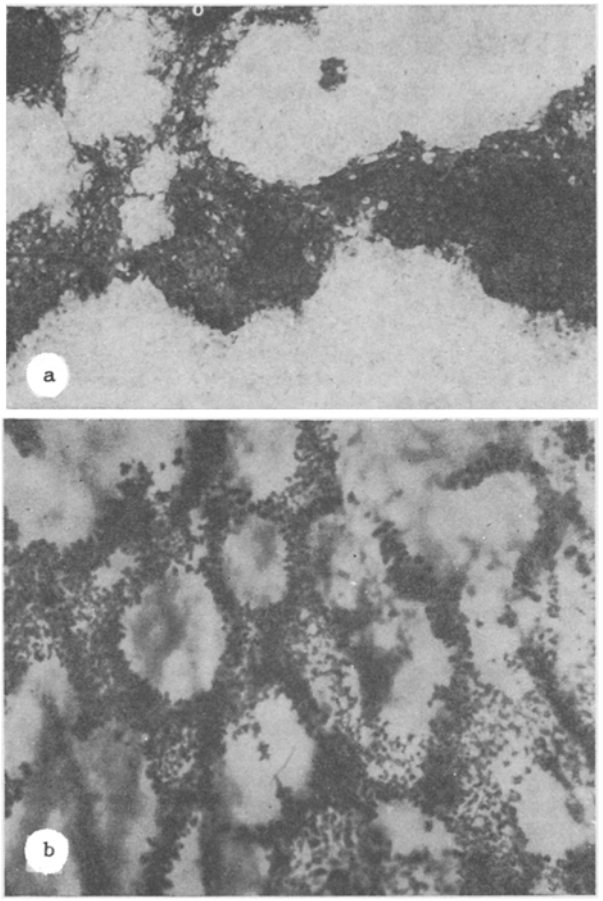

Fig. 1. Total preparations of bone marrow cultures. a) 20 days: deposition of calcium in ground substance of bone around immured osteoblasts (objective 40, ocular 10); b) 22nd day: layer of mineralized bone tissue formed on surface of filter (objective 9, ocular 10). Von Kossa's reaction.

However, osteogenesis as a rule does not develop if bone marrow is explanted *in vitro*. Meanwhile strains of bone marrow fibroblasts obtained in cultures preserve their osteogenic potential even after long-term culture, as is shown by bone and cartilage formation by cells of these strains on retransplantation *in vivo* [7-10].

The aim of this investigation was to study bone formation in organ cultures of adult mouse bone marrow.

EXPERIMENTAL METHOD

The contents of the medullary cavity of the femoral diaphyses of (CBA × C57BL)F_1 mice weighing 18-20 g were expelled by a syringe into medium 199, after both epiphyses had previously been cut off. The bone marrow was expalnted in the form of tissue fragments comprising the contents of one-quarter, one-half, or the whole of a medullary cavity. Explantation was carried out by the method of multiple organ cultures on millipore filters in Conway flasks [1]. HA filters (pore diameter 0.45 μ) with an area of 64 mm^2, numbering 10 per dish, were placed above the round holes in a plastic support so that the lower surface of the filters was moistened with culture medium, and the upper surface was in contact with the gaseous phase. The explanted tissue fragment of bone marrow or spleen (control) was placed on the upper surface of the filter. Culture was carried out on medium α-MEM with 20% bovine embryonic serum and with the addition (per 100 ml of medium) of 15 mg vitamin C, 50 mg L-glutamine, 400 mg glucose, and 6000 U each of penicillin and streptomycin. The gaseous phase consisted of 5% CO_2 in air. The medium was changed every 3 days. After the 8th day of culture, sodium β-glycerophosphate was added to the medium of some cultures in a concentration of 10 mM.

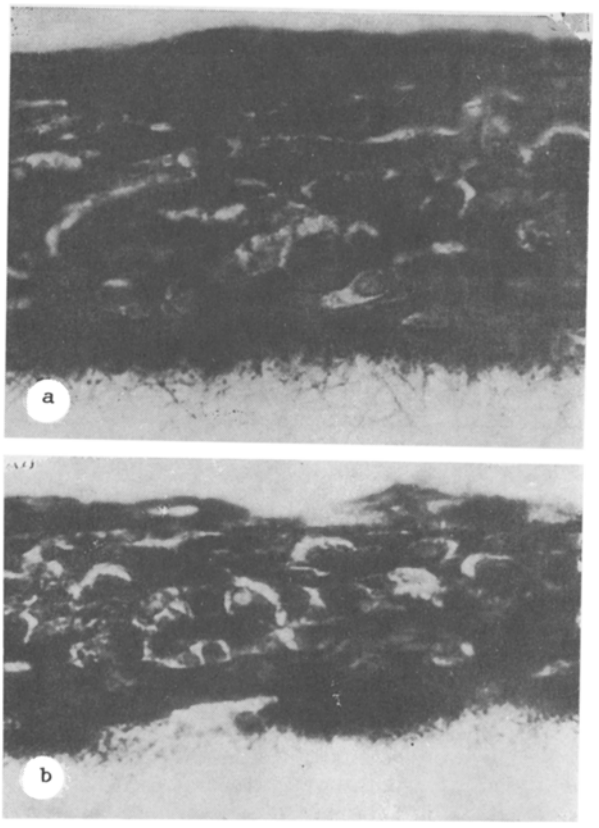

Fig. 2. Sections through bone marrow cultures. a) 26 days: positive reaction for alkaline phosphatase in bone tissue and tissue surrounding it and in zone of filter into which processes of lower layer of osteoblasts penetrated; b) 26 days: positive reaction for alkaline phosphatase; foci of phosphatase activity in regions of filter beneath zone. Objective 25, ocular 10. Gomori's reaction, counterstained with hematoxylin.

The cultures were fixed on the 6th-36th day. For light microscopy, filters with cultures were fixed with alcohol-formol or with 96% ethanol and treated histologically: some cultures were distributed into series of paraffin sections, from others total preparations were made. The sections were stained for calcium by Von Kossa's method, for alkaline phosphatase by Gomori's method, and with hematoxylin and eosin. The total preparations were stained by Von Kossa's method and counterstained with hematoxylin. Filters with cultures were fixed for electron-microscopy with 3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed with 2% OsO_4, dehydrated in alcohols, and embedded in Araldite. Ultrathin sections were stained with lead acetate by Reynolds' method.

EXPERIMENTAL RESULTS

After the first few days of culture of explanted bone marrow fragments the filters were covered with macrophages and myeloid cells migrating from the fragment. Later, proliferation of fibroblasts began on the filter. Near the fragment they formed a stratified zone of growth, but at the periphery of the filter they grew as a single-layered network. Gomori's reaction was positive in the fibroblasts, and more strongly so in those located in the stratified layers. Toward the end of the 2nd week the number of hematopoietic cells within the fragment and in the zone of growth decreased sharply. A dense layer of fibroblasts formed in the center of the culture, with macrophages on its surface. Thin trabeculae and bands of ground substance of bone formed in the composition of the stratified zone of fibroblasts in the course of the 3rd week, and cells with the morphology of osteoblasts were arranged on their surface in the form of a palisade. Gomori's reaction was strongly positive both in the ground substance and in the cells surrounding it.

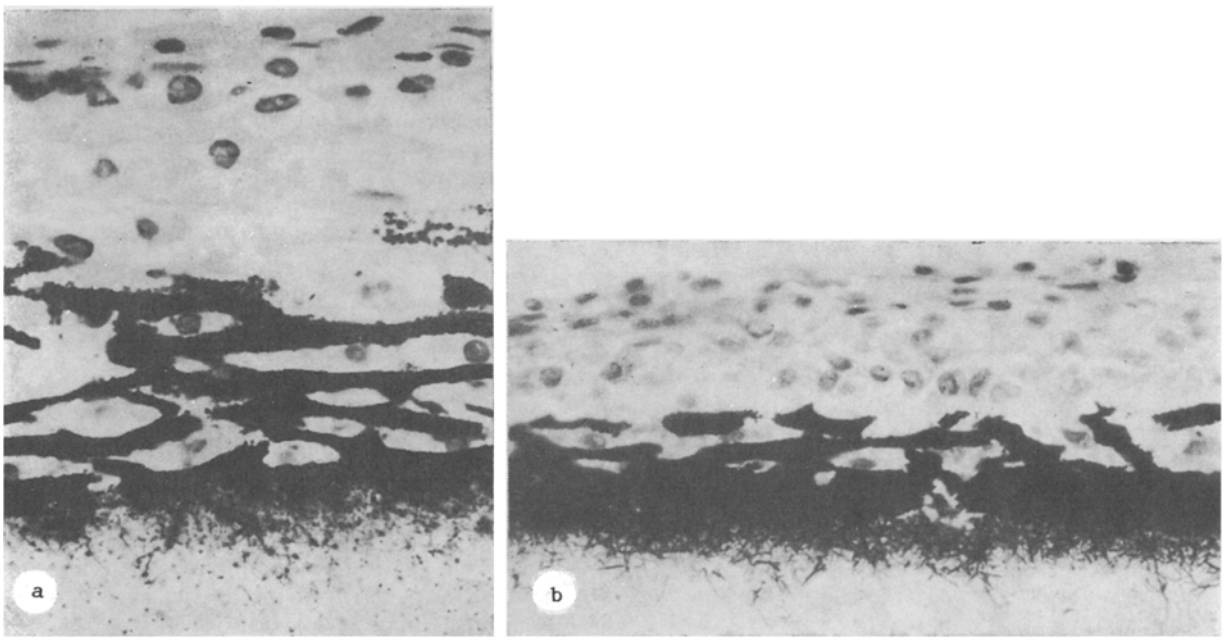

Fig. 3. Sections through bone marrow cultures. a) 26 days: trabeculae of mineralized bone tissue in part of culture adjacent to filter; b) 22 days: mineralized lamina of bone on surface of filter, deposits of calcium along processes of osteoblasts in pores of filter, layer of osteoblasts adjacent to bone trabeculae in course of construction. Objective 25, ocular 10.

If glycerophosphate was added to the culture medium, insoluble calcium began to appear after the 16th day in the trabeculae. As a result the osteoblasts appeared to be surrounded by bands of ground substance, and they formed a unique network in total preparations (Fig. 1). During the 4th week of culture the quantity of ground substance increased rapidly and the osteoblasts appeared immured in it. In this way a structure similar to developing dermal bone, which creeps over the surface of the millipore filter was formed. Above, it was covered with a layer of phosphatase-negative macrophages, and the lower layer of osteoblasts, lying next to the filter, had long phosphatase-positive processes, penetrating into the pores of the filter to a depth of up to 40 μ. By the 26th-30th day of culture the lamina of bone covering the filter had reached its full development and consisted of three to five layers of cells, immured in the mineralized ground substance. The reaction for alkaline phosphatase continued to be intensive in the osteoblasts and in the ground substance at the periphery of the lamina, but became weaker in its center (Fig. 2). The whole lamina at this time was undergoing intensive mineralization: Von Kossa's reaction was strongly positive in it (Fig. 3). The substance of the filter around the islets of osteoblasts sinking into it also underwent intensive mineralization. The zone of calcification of the filter reached a depth of 30 μ. At later stages of culture, cells immured in the mineralized ground substance began to reveal degenerative changes.

Bone tissue was formed after explantation of bone marrow fragments of all three sizes used. In the phosphatase-positive zone of growth of fibroblasts in the splenic cultures, neither osteogenesis nor calcification was observed.

The electron-microscopic investigation showed that the bony structurs on the surface of the filter had the morphology of typical bone tissue. This tissue consisted of densely packed collagen fibers, impregnated with needle-shaped crystals of hydroxy-apatite, which in the late stages were distributed all along the collagen fiber, but in the early stages of mineralization, they had the appearance of centers of calcification characteristic of developing bone tissue. The cells immured in the calcified ground substance had the morphology of typical osteoblasts with a developed ergastoplasm. They were separated from the calcified ground substance by a narrow lacuna. Mineral deposits in the depth of the filter beneath the bone had different electron-microscopic characteristics: Here there were no collagen fibers and crystals were not needle-shaped.

In its morphological characteristics, bone tissue which formed in organ cultures of bone marrow was similar to that found *in situ*. In the cultural system suggested, calcification of the newly formed bone took place only when sodium β-glycerophosphate was added to the medium. In its absence, the characteristic morphological structures of bone were formed, including osteoid, although they did not calcify. In cultures of fragments of mouse spleen, in which the connective-tissue zone of growth also had high alkaline phosphatase activity, no hydroxyapatite was deposited despite the presence of glycerophosphate in the medium. This also was true of the zone of alkaline phosphatase activity in the depth of the millipore filters in bone marrow cultures, where mineralization evidently took place due to the formation of calcium carbonate.

The bone which formed in organ cultures of bone marrow had the characteristic shape of a flat plate, covering the surface of the millipore filter. The area of this plate depended on the size of the original explant.

Compared with cultural systems used previously for explantation of osteogenic cells, organ cultures of bone marrow enable more complete differentiation of bone to be obtained; in other systems only the individual stages of histogenesis of bone tissue could be reproduced, not the process as a whole. The results described above show that medullary osteogenic precursor cells do not need any epigenomal factors in order to form bone tissue other than those which act in culture, including growth-stimulating factors of embryonic serum. The completeness of histogenesis of bone in the organ cultures and the simplicity of this system compared with that *in vivo* make it attractive for the study of the mechanisms of osteogenesis and of problems connected with development of bone tissue under normal and pathological conditions.

LITERATURE CITED

1. E. A. Luriya, Hematopoietic and Lymphoid Tissue in Culture [in Russian], Moscow (1972).
2. K. V. Petrakova, A. A. Tolmacheva, and A. Ya. Feidenshtein, Byull. Eksp. Biol. Med., No. 12, 87 (1963).
3. A. Ya. Fridenshtein, A. A. Ivanov-Smolenskii, R. K. Chailakhyan, et al., Exp. Hematol., 6, 440 (1978).
4. A. Ya. Fridenshtein, N. V. Litsinik, A. G. Grosheva, and Yu. F. Gorskaya, Exp. Hematol., 10, 217 (1982).
5. A. Ya. Fridenshtein, K. V. Petrakova, A. I. Kuralesova, and G. P. Frolova, Transplantation, 6, 230 (1968).
6. A. Ya. Fridenshtein, I. I. Pyatetskii-Shapiro, and E. V. Petrakova, J. Embryol. Exp. Morphol., 16, 381 (1966).
7. A. Ya. Fridenshtein, R. K. Chailakhyan, and K. S. Lalykina, Cell Tissue Kinet., 3, 393 (1970).
8. R. K. Chailakhyan, Yu. V. Gerasimov, and A. Ya. Fridenshtein, Byull. Eksp. Biol. Med., No. 11, 605 (1984).
9. B. A. Ashton, T. D. Allen, C. R. Howlett, et al., Clin. Orthop., 151, 294 (1980).
10. H. M. Patt, M. A. Maloney, and M. L. Flannery, Exp. Hematol., 10, 738 (1982).