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Differentiation *in vitro* of Embryonic Cartilage and Bone in a Chemically-Defined Medium.* (30160)

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Fell(1) first reported the cultivation of limb bud mesenchyme of the chick embryo and described osteogenesis *in vitro*; this study has been confirmed by other investigators(2-4). The purpose of the present communication is to report *in vitro* osteogenesis in posterior limb rudiments from 14-day-old embryonic mice. A completely defined chemical medium, containing neither plasma, embryo extract, nor any biologic supplement, was employed.

Materials and methods. Fourteen-day-old mouse (C57BL/6J) embryos were used. The mating system was as follows: (1) males were placed with females at about 4 PM; (2) between 8 and 9 AM the next day, the females were examined for vaginal plugs; (3) most mating occurred between midnight and 2 AM; therefore a "14-day embryo" is actually about 14 days and 8 hours post-mating.

The culture medium MAB87/3 (Table I) used in these experiments is one designed by one of us (C.W.) primarily for establishment of cultures from freshly explanted cells and tissues. The only notable way in which it differs from media designed for cell strains, *e.g.*, medium MD705/1(5), is in the inclusion of insulin. Other differences are mainly quantitative.

The entire embryo was separated from the uterus and fetal membranes and immersed promptly in Hanks' solution(6) at room temperature. After the embryos were removed from this solution, a posterior limb was dissected from each. This was then placed in a small drop of MAB87/3 culture medium on a tissue culture coverglass (3 × 0.7 cm). The coverglass was introduced into a 3.5 cm diameter Carrel flask (containing 1 ml of the liquid medium) which was closed by a sterile rubber stopper. The flasks were incubated at 37°C for periods of 3, 5, and 12 days. The culture medium was changed every 4 or 5 days. At the end of the appointed time of incubation, the limb was placed in Bouin's fixative, dehydrated and embedded in paraffin. Serial sections of the undecalcified limbs were cut at 10 μ. They were stained with hematoxylin and eosin, or with the periodic acid-Schiff stain.

Four embryos of the same age from the same litter served as controls. Without dissection, the entire embryos were placed in fixative and prepared histologically like the experimental series.

Results. Serial sections of the limbs from the controls showed the bone primordia to be composed of only cartilage. No collagen, osteoid or osteocytes were visible by trans-

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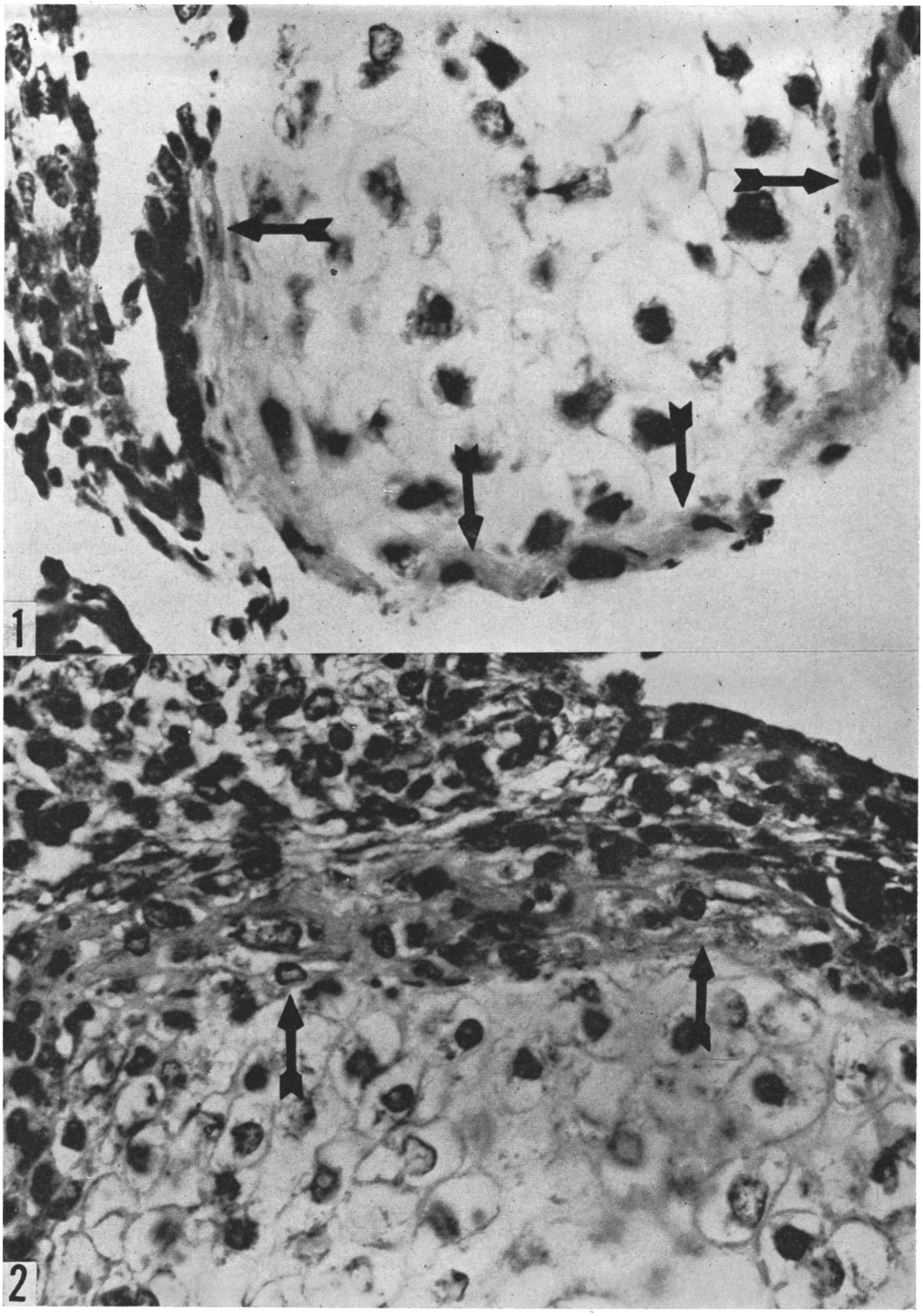

FIG. 1. Photomicrograph of a section of embryonic mouse tibia grown in tissue culture

medium MAB87/3 for 5 days. Section shows a thin rim of perichondrial new bone in sleeve bone area (arrows). ×144. Hematoxylin and eosin.

FIG. 2. Photomicrograph of a section of embryonic mouse femur grown in tissue culture medium MAB87/3 for 12 days. Section shows a marked increase in perichondrial new bone in sleeve bone area (arrows). ×144. Hematoxylin and eosin.

mitted light, but with the aid of phase contrast and polarized light, a few collagen fibers could be seen at the periphery of the cartilage in the sleeve bone area.

After 3 days incubation in medium MAB87/3, limbs showed only cartilage in the primordial bones. It was usually possible to identify cartilagenous areas destined to become pelvis, femur, tibia and fibula.

When sections of the controls and the 3-day cultured embryos were compared under phase contrast and polarized light those from the latter showed a marked increase in collagen. No osteoid or bone was observed in either group.

Sections of an embryo cultured for 5 days disclosed a rim of newly formed perichondrial bone (Fig. 1) that contained osteocytes. Examination of the sections by polarized light was confirmatory.

After 12 days in culture, sections of the limbs showed a further increase in the amount of new bone (Fig. 2). Sections treated with periodic acid-Schiff stain also showed the presence of new bone.

Discussion. Biggers(7) and his associates reported the growth of 15-day-old mouse embryo tibiae in a relatively simple, chemically-defined medium. They observed an increase in length, net weight, and dry weight. Histologic sections were not made. Ossification centers do not appear in the mouse tibia until the embryo is more than 14 days of age (8-10). Meng(9) is the only investigator who has documented his findings with serial, transverse sections of the embryos. In 16-day embryos, he observed a thin tube of bone around the cartilagenous tibia. The subperiosteal vascular connective tissue irrupts into the calcified center of the cartilagenous diaphysis 24 hours later.

Ghisellini and Ladu(11) studied the effect of gamma radiation on the development of cartilage in 12-day-old mouse embryos grown in conventional tissue culture media. After 3 days of culture they found that the anterior limbs showed normal patterns of development comparable to those observed in normal intact embryos. The cartilage cells in the metacarpal outline contained large, round nuclei and were separated from one another by a small amount of ground substance. They did not attempt to show that osteogenesis would occur if the embryos were cultured for a longer period.

As Shaw and Bassett(12) have pointed out, specific advantages and disadvantages are attached to the use of whole organ, or

TABLE I. Chemically Defined Medium MAB87/3.

Ingredient	mg/100 ml	Ingredient	mg/100 ml	Ingredient	mg/100 ml
NaCl	600	Glutathione	1.5	L-glutamic acid	15.0
KCl	15	Hypoxanthine	2.5	L-serine	12.8
$CaCl_2 \cdot 2H_2O$	12	Thymidine	8.0	L-alanine	11.2
$MgCl_2 \cdot 6H_2O$	24	Choline • HCl	25.0	L-arginine • HCl	7.5
$MgSO_4 \cdot 7H_2O$	10	Insulin*	.8	L-threonine	7.5
Na_2HPO_4	30	Thiamin • HCl	1.0	L-valine	6.5
KH_2PO_4	20.8	Ca pantothenate	.1	L-aspartic acid	6.0
$NaHCO_3$	224	Riboflavin	.1	Glycine	5.0
$FeSO_4$	.045	Pyridoxin • HCl	.1	L-proline	5.0
$CuSO_4 \cdot 5H_2O$	.005	m-Inositol • $2H_2O$	.1	L-leucine	5.0
$MnSO_4 \cdot H_2O$	.0016	Nicotinamide	.1	L-methionine	5.0
$ZnSO_4 \cdot 7H_2O$	.003	Vitamin B_{12}	.02	L-phenylalanine	5.0
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	.0025	Folic acid	.05	L-tryptophan	4.0
$CoCl_2 \cdot 6H_2O$	.0022	Biotin	.002	L-tyrosine	4.0
Glucose	500	L-glutamine	35.0	L-isoleucine	2.5
Ascorbic acid	1.75	L-lysine • HCl	24.0	L-asparagine	2.4
Cysteine • HCl	9.0	L-histidine • HCl	15.0	L-cystine	1.5

* Gifts of amorphous insulin (Lilly) from Dr. Otto Behrens and Dr. William W. Brower, Lilly Research Laboratories, are gratefully acknowledged.

endosteal cell, cultures in the study of osteogenesis. The latter is difficult and time-consuming; in the former it may be hard to distinguish the new bone formed during culture from that present before culture. In spite of this problem, it was decided to study the growth of embryonic cartilage and bone in organ culture in a new, completely-defined chemical medium. The results obtained justify the choice. Several attempts have been made to duplicate Fell's work using a completely-defined chemical medium instead of a plasma clot. Except in one instance(15), other investigators(13,14) found that biologic supplements had to be added to the medium in order to observe osteogenesis in cultured limbs. Past(15), in reporting the results of a study of Fe^{59} uptake by bone primordia, observed osteogenesis in the limbs of mouse embryos which were cultured in a protein-free, chemically-defined medium(16). The medium which he employed (NCTC 109) is more complex than that used in the experiments reported herein.

Summary and conclusions. 1. Fell first reported osteogenesis in chick embryo limb buds cultured *in vitro* in a plasma clot in 1928. Since that time several attempts to repeat her work using a completely-defined chemical medium have failed. Except in one instance, success was achieved only when biologic supplements, *e.g.*, embryo extract or bovine serum albumin, were added to the medium. 2. This communication reports differentiation of embryonic cartilage and bone of the mouse *in vitro* using a new, completely-defined chemical medium (MAB87/3) which is the simplest thus far reported. Biologic supplements were not necessary. 3. The 14-day embryo limb rudiment develops a rim of perichondrial bone after 5 days of culture in this medium. A further increase in amount of new bone was visible after 12 days of culture.

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