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The Matrix of Rat Calvarium as Transformant of Fibroblasts[1] (39028)

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On allogeneic transplantation demineralized matrix of rat bone transforms responding fibroblasts to chondroblasts and osteoblasts (1); hemopoietic bone marrow forms in the new ossicle (2). Transformation of this sort is critically dependent on the geometry and surface charge (3–5) of the transformant and on the site where the ossicle (2) is created. The transforming potency varies widely in the matrices of different bones of an individual rat; the demineralized residues of tubular bones are more active than the matrices of flat bones (5). In the present experiments, the transforming competence of matrices of rat calvarium and diaphysis was evaluated on samples tested singly and in mixtures, respectively. It was found that calvarial preparations depressed the efficiency of transformability of diaphyseal matrix.

Materials and methods. Bioassay of transformation. The frontal and parietal bones of the calvarium of adult rats, dissected free of periosteum and bone marrow, were washed in a jar of distilled water with the aid of a magnetic stirrer. Fragmented chips of the calvarium were used to prepare the demineralized matrix essentially as described before (1). Diaphyseal bone matrix prepared concurrently served as control. In experiments designed to evaluate the influence of mixtures of the two osseous matrices, weighed amounts in designated ratios were thoroughly mixed by shaking prior to transplantation.

Male and female rats of Long–Evans strain, age 28–35 days, were employed. The matrices under investigation were transplanted (25–30 mg per site) to eight subcutaneous sites in the trunk at four symmetric locations: upper and lower thoracic, abdominal and dorsal. On designated days of harvest $^{45}CaCl_2$ in saline was injected via a caudal vein at a dose of 1 μCi/g body weight 4 hr prior to termination of the experiment. At harvest, the transplantation plaque was weighed and portions were homogenized for alkaline phosphatase determination and ^{45}Ca incorporation as described earlier (1). One unit of alkaline phosphatase (EC 3.1.3.1) is defined as the enzyme activity which liberates 1 μmole of *p*-nitrophenol per 0.5 hr under stated conditions (6). Portions of the plaque were fixed in Bouin's fluid or in neutral formalin for histological examination; paraffin sections were stained with hematoxylin-eosin. Sections of neutral formalin-fixed tissues were stained additionally with $AgNO_3$ to reveal foci of calcification.

Results. On transplanting the calvarial bone matrix to subcutaneous spaces there ensued a sequence of interconnected biological responses culminating in the formation of bone and bone marrow. However, the yield of bone as assessed by the triad of alkaline phosphatase, ^{45}Ca incorporation and histology was considerably less than that elicited by diaphyseal bone matrix (Table I).

On day 1 the transformation plaque was discrete and encapsulated. Histological examination revealed that the mass was a conglomerate of the transformant matrix, fibrin and polymorphonuclear leukocytes. It is well known that alkaline phosphatase is a characteristic constituent of the polymorphonuclear leukocytes (7) and it was useful in the present investigation to quantitate the leukocyte emigration elicited by the bony matrices. Based on the alkaline phosphatase activity, it would appear that the leukocyte-response was greater to the calvarial matrix. Additionally, we demon-

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TABLE I. CHANGES IN ALKALINE PHOSPHATASE ACTIVITY AND ^{45}Ca INCORPORATION IN PLAQUES ELICITED BY CALVARIAL AND DIAPHYSEAL BONE MATRIX.

	Calvarium			Diaphysis		
Day	Alkaline phosphatase (units/g)	^{45}Ca (cpm/mg)	Histological appearance	Alkaline phosphatase (units/g)	^{45}Ca (cpm/mg)	Histological appearance
1	10.6 ± 1.0	N.D.[b]	PMN[c] ++++	2.9 ± 0.5	N.D.	PMN +
3	8.9 ± 0.3	22 ± 4[a]	PMN ++++	4.5 ± 0.4	31 ± 10	PMN + and fibroblasts ++
5	3.6 ± 0.4	55 ± 20	PMN + and fibroblasts ++	2.7 ± 0.3	32 ± 5	Fibroblasts ++++
7	2.9 ± 0.3	108 ± 40	Fibroblasts ++++	15.3 ± 1.3	80 ± 17	Cartilage ++
9	4.0 ± 1.1	25 ± 6	Cartilage +	27.3 ± 4.0	1893 ± 682	Cartilage ++++
11	3.8 ± 0.3	674 ± 320	Cartilage +	63.6 ± 9.3	6461 ± 908	Calcified cartilage ++++ bone ++
14	4.2 ± 0.7	150 ± 80	Cartilage +	65.9 ± 3.5	8109 ± 912	Bone ++++
18	3.7 ± 0:8	408 ± 132	Bone trace	46.9 ± 3.7	8049 ± 1115	Bone ++++
21	9.3 ± 2.5	2357 ± 714	Bone +	29.8 ± 2.6	7004 ± 459	Bone ++++ Bone marrow ++++
28	6.9 ± 1.3	2198 ± 344	Bone + Bone marrow trace	20.9 ± 2.6	6065 ± 440	Bone ++++ Bone marrow ++++
35	6.5 ± 1.7	1057 ± 400	Bone +	13.5 ± 1.7	5769 ± 203	Bone ++++ Bone marrow ++++
50	9.9 ± 1.8	2226 ± 627	Bone ++ Bone marrow trace	16.7 ± 1.5	7662 ± 471	Bone ++++ Bone marrow ++++

[a] S.E. of mean of 8 or 16 observations.
[b] Not detectable.
[c] Polymorphonuclear leukocytes.

strated alkaline phosphatase activity in these leukocytes by a histochemical technique (8). Whereas the leukocytes persisted as long as day 5 in the plaques elicited by calvarial matrix, in the diaphyseal matrix they disappeared on days 2–3. The leukocytes were transient as indicated by declining alkaline phosphatase activity and histological appearance. On day 5 fibroblasts were seen in close proximity to the implanted matrices. On day 7 considerable amounts of cartilage were seen in the transformation plaques of the diaphyseal matrix. The appearance of cartilage was delayed in the plaques of the calvarial matrix and the yield was scant and not distributed uniformly in contrast to the long bone matrix. It is noteworthy that cartilage was confined to core and was never observed in the rim of the plaque. By days 9–11 the incursion of capillaries resulted in chondrolysis and the appearance of osteoblasts and new bone formation on the surface of the matrix by appositional growth. The *de novo* osteoblastic activity correlated with a marked increase in ^{45}Ca incorporation into bone mineral and the values attained a plateau by day 14. However, the appearance of bone and later bone marrow in the plaques was retarded in the case of calvarial matrix and quantitatively was lower than the plaques in response to diaphyseal bone matrix. The quantitative difference between the ossicles created by the calvarial and diaphyseal bone matrix was sustained and persisted throughout the experimental duration. In other experiments we observed that the transformation ossicles created by diaphyseal matrix were vivid red in color from day +18 onwards; in contrast, the calvarial matrix-induced ossicles were not red in the gross and myelopoiesis was sparse.

In view of the observed weak transforming potency of the calvarial matrix, it was of interest to investigate the influence of this matrix on the response obtained by diaphyseal bone matrix. The results of a typical

TABLE II. INFLUENCE OF MIXTURES OF BONE MATRICES FROM CALVARIUM AND DIAPHYSIS ON TRANSFORMATION OF FIBROBLASTS.

Ratio of matrix by weight (%)		Alkaline phosphatase (units/g)	^{45}Ca Incorporation (cpm/mg)	Histological appearance
Calvarium	Diaphysis			
100	—	3.4 ± 0.3[a]	129 ± 64	Cartilage traces
75	25	11.3 ± 1.7	204 ± 102	Cartilage +
50	50	11.2 ± 1.7	196 ± 115	Cartilage ++
25	75	22.5 ± 4.2	225 ± 62	Calcified cartilage ++
—	100	65.6 ± 8.4	1864 ± 710	Calcified cartilage ++++

[a] SE. of mean of eight observations.

experiment are depicted in Table II. It reveals that addition of 25% by weight of calvarial matrix to the diaphyseal bone matrix resulted in a significant decrease ($P < 0.01$) of alkaline phosphatase activity and ^{45}Ca incorporation in the transformation plaques on day 9.

Discussion. Calvarial bone matrix is competent to transform fibroblasts; albeit the yields are lower as compared to diaphyseal bone matrix. It is noteworthy that whereas in the ontogeny of calvarium there is no cartilage stage, the calvarial matrix transformed fibroblasts to chondroblasts.

In the transformation of the sort described in this paper, the matrix has two important attributes: chemotaxis and transforming potency. The present results show that the chemotaxis for leukocytes was greater in the calvarial matrix than the diaphysis. In contradistinction, the transforming potency was higher in the diaphyseal bone matrix. The leukocyte chemotaxis was a transient event whereas the fibroblast chemotaxis was longer in duration and resulted in transformation to new phenotypes on a defined temporal sequence (1).

The reasons for the marked differences in the transforming ability of the two osseous matrices are not clear at present. It is possible that the collagen fiber orientation in the weight-bearing long bones is different from calvarial bone and this may confer different geometric and surface characteristics on matrix.

Summary. Allogeneic transplantation of coarse powders of the rat calvarial matrix to subcutaneous spaces resulted in transformation of fibroblasts to chondroblasts and osteoblasts. The emergence of new phenotypes was quantitated by determination of alkaline phosphatase activity and ^{45}Ca incorporation. A comparison of the matrices from rat calvarium and diaphyseal bone revealed that matrix of the shaft of long bones is more potent as transformant than matrix of the vault of the skull.

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