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ON THE OCCURRENCE IN VITRO OF CELLS RESEMBLING OSTEOCLASTS

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During the early stages of the establishment of a 'pure' strain of endosteal fibroblasts in vitro, it was observed that a number of actively motile giant cells of unusual type were appearing fairly regularly among the cells growing out from explanted pieces of embryonic bone. As no description of similar cells in vitro could be found in the literature it was decided to investigate their properties, and, if possible, their source.

MATERIALS AND METHODS

The usual routine for hanging-drop tissue culture was employed; small fragments of membrane bone, obtained from the frontal bone of 13-day chick embryos, were explanted on to coverslips in a cock-plasma clot. After incubation for the requisite period, the cultures were fixed in Carnoy's fluid and stained subsequently either with carmalum, Meyer's haematoxylin and eosin, or Heidenhein's iron haematoxylin. The cultures were mounted in toto in balsam.

RESULTS

Examined after 24 hr. incubation, the cultures showed the usual outgrowth of an actively proliferating inner zone of spindle-shaped fibroblasts, and an outer zone comprising wandering cells which were both microphage and macrophage in type (Pl. 1, fig. 1). The former appeared to be heterophil granular leucocytes and were mostly degenerate. Of the latter, some may have come from spindle cells, which had wandered out into the clot; others were similar in appearance and staining reaction to blood monocytes.

In the living cultures, the cells shortly to be given the title of osteoclasts appeared in variable numbers, not as a 'strain' of cells deliberately and preferentially cultured, but as a comparatively small minority in a mixed cell population. They were found after 24 hr. incubation mostly in the outer zone among the wandering cells (Pl. 1, figs. 1, 2), but they did not always become free of the spindle-cell zone and often seemed to be enmeshed in the latter. They appeared before fixation as discrete bodies, characterized by a refractile, granular cytoplasm and standing out from the spindle cells as shining masses. Their shape was too variable for a mean type to be described, but two sub-

divisions were distinguished: one in which the cells, adhering to the underside of the coverslip, were flattened out by contact; the other, in which they were entirely surrounded by the medium, and therefore, if not spherical, at least 'three-dimensional' in appearance. The cells often occurred in groups of two or three. Their long axes were as often transverse as parallel to those of the spindle cells; it was not possible to follow their mode of progression, but it is hoped to do so with the aid of microcinephotography at a later date. The many nuclei could be seen in the living cell. The cell boundary was clear-cut and the outlines of the pleomorphic pseudopodia were fairly easy to follow. Many intracytoplasmic vacuoles were present in the larger cells (vide infra).

Observations on fixed and stained material, which it will be convenient to discuss under the following headings, confirmed and amplified the above findings.

Shape and size of cell

As measurement in one plane only was practicable, attempts to assess cell size accurately were abandoned. At one end of the scale were individuals little larger than a monocyte, containing one, two or more nuclei, and, at the other end, those of enormous proportions, with axes measuring up to 120μ and containing as many as 110 nuclei. The apparent size of a small spherical cell increases when it flattens itself against the coverslip, but 'flattens' is a relative term. In certain extreme cases the cell in contact with the glass was so attenuated that its nuclei became packed together in box-like form, the cell itself being invisible except through a $\frac{1}{12}$ in. objective (Pl. 1, fig. 3).

The cells varied greatly in size and functional activity; pseudopodia of varying appearance occurred, and some were occupied by one or more nuclei. Dumb-bell-shaped cells were seen, consisting of two multinucleate masses connected by a thin cytoplasmic strand. Whether they were in process of separating or fusing, or whether this appearance resulted merely from an exuberance of pseudopodial activity is impossible to say without the assistance of microcinephotography (Pl. 1, fig. 4).

Staining reaction

The outstanding characteristic observed was the presence of an inner, chromophil cytoplasmic zone merging into an outer, chromophobe zone. This was sometimes wavy and crenellated in outline. These zones may correspond respectively to an outer, tenuous and fluid exoplasm—the undulating membrane—and the inner, more substantial gelled layer of endoplasm. The exoplasm was itself sometimes ringed by a fringe of chromophil cytoplasm (Pl. 1, fig. 5).

The cytoplasm was strongly oxyphil and finely granular, but the fixing agent employed may have determined the granularity. The smaller types, containing one or two nuclei, often appeared more oxyphil than their larger relatives, probably because they were also frequently more spherical.

Multinuclearity

Regularity in the shape and size of the nuclei was a characteristic feature (Pl. 1, figs. 3, 5 and 6). The average nucleus was spherical or slightly ovoid, with a diameter of 3·5μ. It was markedly basophilic. Rather reticular, it generally possessed one clear-cut nucleolus; more rarely, two were seen. The plastic nature of the nuclei was apparent in cells in which they were tightly packed. In the average cell, they were irregularly disposed, generally within the chromophil endoplasm. The average number per cell (estimated on a basis of approx. 1000 cells) was about six, with a highest recorded maximum of 110.

Many cells with similar staining reactions, and with well-marked exo- and endoplasm, were seen, little larger than a monocyte (and indeed frequently no larger) which contained one, two or three nuclei (Pl. 2, figs. 7, 8). These are regarded as immature individuals of the same series.

General observations

On no occasion was either mitotic or amitotic division seen, and, conversely, at no time were spindle cells seen fusing to produce fresh multinucleate masses. No fragments of bone matrix were observed within the cells or free within the medium, so that it is unlikely that the cells had transported any such into the clot. At times, however, refractile brownish crystals of undetermined origin, and at times erythrocytes, were seen within the cytoplasm.

After 48 hr. the cells were degenerate; the cytoplasm was coarsely reticular, intensely eosinophilic, and loaded with vacuoles. The nuclei were shrunken, of bizarre shape, and pyknotic. Soon after, little remained save an unrecognizable amorphous mass of granular oxyphil material.

Examination of cultures in the early stages of growth showed that the giant cells originated as such from the bony fragment, that they appeared well in advance of the outgrowth of spindle cells, and that they continued to wander out from the explant into the medium at various times after incubation began. The earliest giant cell seen emerged from the cut surface of the bone at the sixth hour, about three-quarters of its body being free within the medium. Within another 4 hr. it had wandered out, completely free. At the other extreme, cells could be seen (much more easily in the fixed than in the living culture) still very near the edge of the bone, as late as the 24th hour.

Having emerged, the cells wandered irregularly outwards from the implant. In their wake they left a track of digested or liquefied plasma (Pl. 2, figs. 9, 10). This point was demonstrated rather strikingly by direct fixation of specimens in Carnoy's fluid, without previously soaking in buffered saline to reduce the stainability of the plasma clot. This unusually vigorous liquefying property may have some functional significance.

It is, therefore, reasonable to assume that these cells are derived from bone and are osteoclasts. That they are not megakaryocytes is evident from the morphology of their nuclear substance. These cells are multinuclear; the megakaryocyte, polymorphonuclear.

DISCUSSION

In so far as no particulate matter resembling bone matrix was seen within the cells, nor free within the medium, the present experiments support the views of McLean & Bloom (1941) that osteoclasts have no osteolytic properties. On the other hand, the plasma-digesting property described does seem to point to possible proteolytic activity, and ingested intracytoplasmic collagenous material is notoriously difficult to demonstrate histologically (Vassos, 1940).

The origin of osteoclasts forms the subject-matter of a considerable literature. The views of the earlier workers have been summarized by Arey (1919), Ham (1932) and Bruno (1937). It may be said in general terms that some (Kolliker, 1873; Howell, 1890; Arey, 1919; Ham, 1932; Bloom, Bloom & McLean, 1941) regard the osteoblast as the source cell from which fresh osteoclasts derive; others, the marrow reticulum-cell directly (Jordan, 1921) or indirectly (Bloom, Bloom & McLean, 1941), whilst others (Mallory, 1911; Haythorn, 1929) favour the monocyte. It may be pointed out, however, that the close relationship between the marrow reticulum cell, the marrow endothelial cell and the monocytes tends to minimize the distinction between these views. The present observations support the last school. First, all stages were seen between cells resembling monocytes in appearance, and the fully developed osteoclast. Secondly, there is in the present series of experiments evidence of a functional nature. In the past, the derivation of osteoclasts seems to have been considered without realization of their vigorous motility; thus the only record of direct experimental work on this point which has been found is that of Kolliker (1873) who failed to observe motility in warm-stage preparations. It is difficult to believe that the mere coalescence of several relatively poorly motile individuals, such as reticulum cells, osteoblasts or osteocytes, could confer upon the resultant mass the property of great motility, which in the case of osteoclasts resembles that of monocytes rather than that of osteoblasts or other 'connective tissue' cells. Finally, if the tenuous exoplasm described above be accepted as the probable site of an 'undulating membrane'—a reasonable assumption—then, here, in the osteoclast, is another analogue of a monocytic characteristic.

The present experiments suggest that the cells arise by simple fusion and not by nuclear division, since no mitotic or amitotic division was observed in roughly a thousand nuclei scrutinized. They shed no light on the problem as to whether osteoclasts are directly responsible for bone resorption.

Although it would be unjustifiable to compare too closely conditions in vitro with those obtaining in vivo, the fact that all osteoclasts observed in vitro were

grossly degenerate within 48 hr. indicates that their life should be measured in terms of hours rather than days. This is in accordance with the findings of Bloom *et al.* (1941). Working with material obtained from pigeons at different stages in the reproductive cycle, these authors describe periods of intense osteolysis which last for only a few hours and which are followed by periods of equally intense bone deposition. Their belief that in these circumstances osteoclasts are transformed into osteoblasts and marrow reticulum cells, and vice versa, is, however, not in accord with the present work.

No conclusions as to the fate of osteoclasts can be drawn from the present work. Although the description by Bloom *et al.* of a chromophil cytoplasmic zone round the osteoclast nuclei is confirmed, no support has been found for their suggestion that this zone separates into nucleated individuals. No budding off or splitting of exoplasm has been encountered, and their Pl. 2, fig. 2 can be interpreted in the light of the present experiments as showing merely an osteoclast in which the inner chromophil cytoplasmic zone is well demonstrated.

SUMMARY

1. In a proportion of cultures of embryonic chick bone, large multinucleated giant cells wander out from the explant.

2. These cells are considered to be osteoclasts and are derived from monocytes by fusion.

3. In vitro, they do not become transformed into other cell types.

It is regretted that precise magnifications cannot be given for the microphotographs which were taken in 1939. The optical bench and records were subsequently destroyed by enemy action and it has not been possible to estimate the bellows extension used. Acknowledgements are due to Mr R. Harrison for assistance with the microphotographs.

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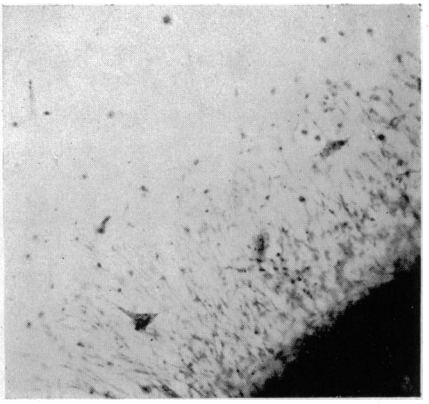

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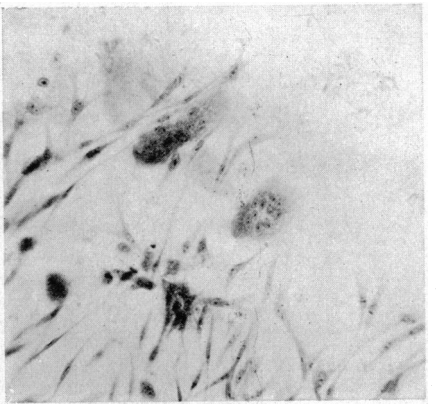

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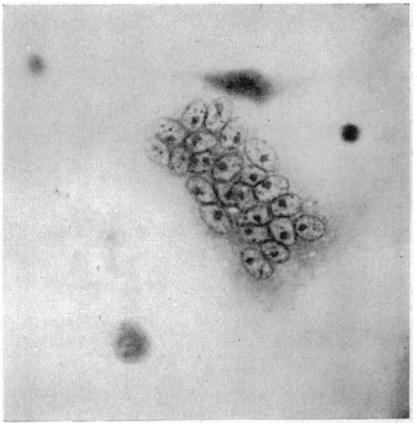

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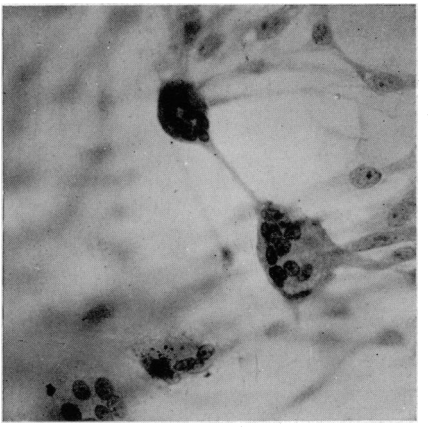

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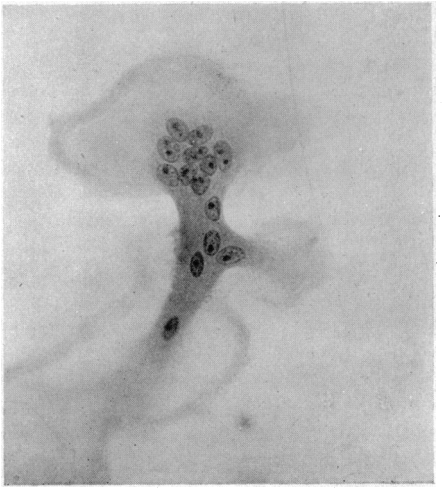

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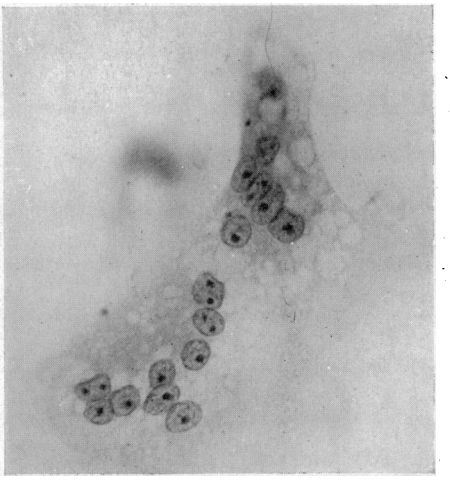

Fig. 6.

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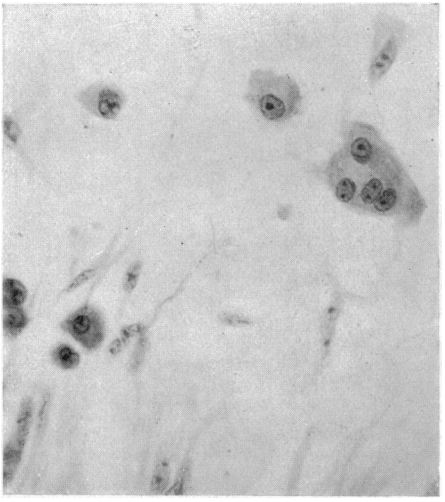

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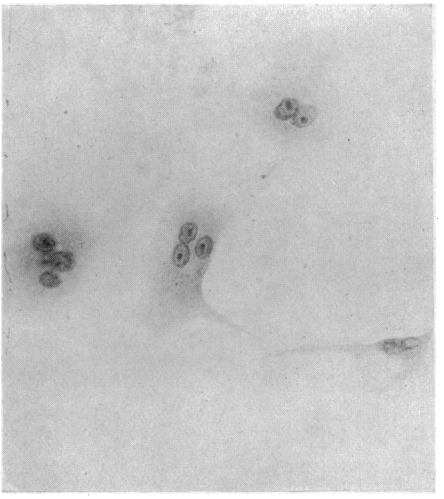

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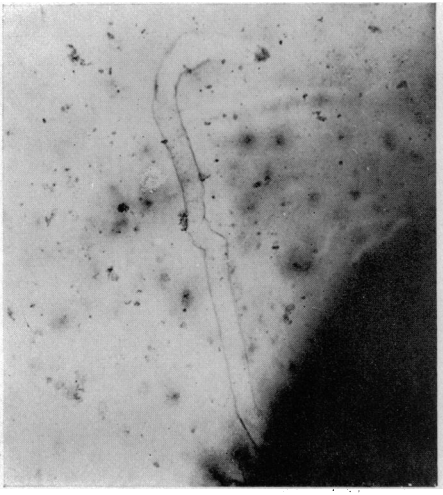

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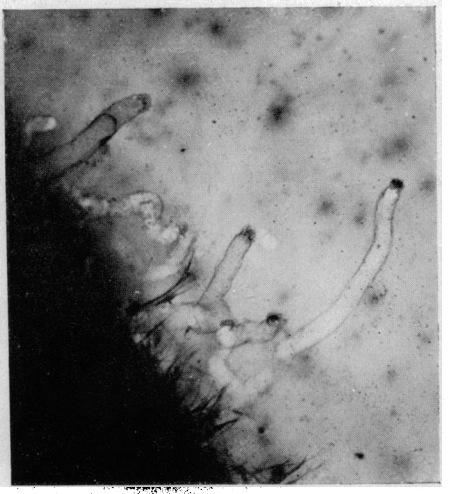

Fig. 10.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. 16 mm. objective, ×4 eyepiece. Edge of bone explant visible; three osteoclasts present in the spindle-cell outgrowth zone. 24 hr. culture.

Fig. 2. 8 mm. objective, ×2 eyepiece. Osteoclasts amidst spindle-cell zone. 24 hr. culture.

Fig. 3. 2 mm. objective, ×4 eyepiece. An osteoclast flattened against coverslip; nuclei pressed together in box-like fashion. 24 hr. culture.

Fig. 4. 4 mm. objective, ×4 eyepiece. An osteoclast in which two roughly spherical masses are connected by a thin cytoplasmic strand. 24 hr. culture.

Fig. 5. 3 mm. objective, ×4 eyepiece. The outer chromophobe and inner chromophil zones. 24 hr. culture.

Fig. 6. 2 mm. objective, ×4 eyepiece. Cytoplasmic vacuolation. 24 hr. culture.

PLATE 2

Fig. 7. 3 mm. objective, ×4 eyepiece. An osteoclast containing four nuclei. Several individuals with a single nucleus can be seen free and rounded within the clot. These are regarded as precursor cells. 24 hr. culture.

Fig. 8. 3 mm. objective, ×4 eyepiece. Osteoclasts containing two, three and four nuclei. 24 hr. culture.

Figs. 9, 10. 16 mm. objective, ×4 eyepiece. Tracts of liquefied plasma clot leading outward from the fragment of bone toward the periphery. Osteoclasts visible at the blind terminations.