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Study of cell kinetics within evolving secondary Haversian systems*

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INTRODUCTION

Evolving secondary Haversian systems, which in larger animals serve to substitute *woven* for *lamellar* bone during growth and to restructure the cortex throughout life (Amprino & Bairati, 1936; Frost, 1963; Johnson, 1964) constitute an interesting model for the study of various aspects of bone cell kinetics and of factors which control them.

Such units originate on the surface of the Haversian or Volkmann's canals (Frost, 1963) and, once constituted, move through the bone at the speed of some 40–50 μm per day (Jaworski & Lok, 1972) over a period of several weeks (Frost, 1963; Johnson, 1964), leaving behind new osteons. Throughout their path the same cytoarchitecture is maintained, as shown schematically in Figure 1: osteoclasts arranged in the resorption front (i.e. cutting cone) are followed by capillaries and osteoblasts which deposit bone on the surface of the eroded cavity (i.e. closing cone) (Frost, 1963; Johnson, 1964).

The stable cytoarchitecture of these units as they move through bone implies a coordination of several self-renewing cell populations: osteoclasts, osteoblasts and endothelial cells. Thus, the supply of osteoclasts to the resorption front, the elongation of the capillaries behind the advancing cutting cone and the replacement of osteoblasts which become lining cells or osteocytes embedded in the new bone unit, all require a continuous recruitment of functional cells from the pool or pools of stem cells (Leblond, 1964).

These features of evolving secondary Haversian systems are inferred from the descriptive histology (Frost, 1963; Johnson, 1964) and histomorphometric analysis of undecalcified bone sections sampled by biopsy after the *in vivo* labelling with tetracycline (Frost, 1966; Hattner, Epker & Frost, 1965).

A study of cell kinetics in such sites, however, which requires the use of autoradiographic technique has not been undertaken. Much has been extrapolated to lamellar bone remodelling sites from studies of bone formation and turnover sites in growing small animals (Frost, 1963). While the origin and fate of various bone cells in such sites and in the evolving secondary Haversian system may not differ, the cell population kinetics of the latter can be only studied in intact animals. These systems appear only in larger animals (Enlow, 1962; Frost, 1963) of a certain age (Amprino & Bairati, 1936).

Consequently, we undertook such a study in young adult Beagle dogs, applying the technique of autoradiography to biopsy and autopsy material collected at various times after the injection of tritiated thymidine.

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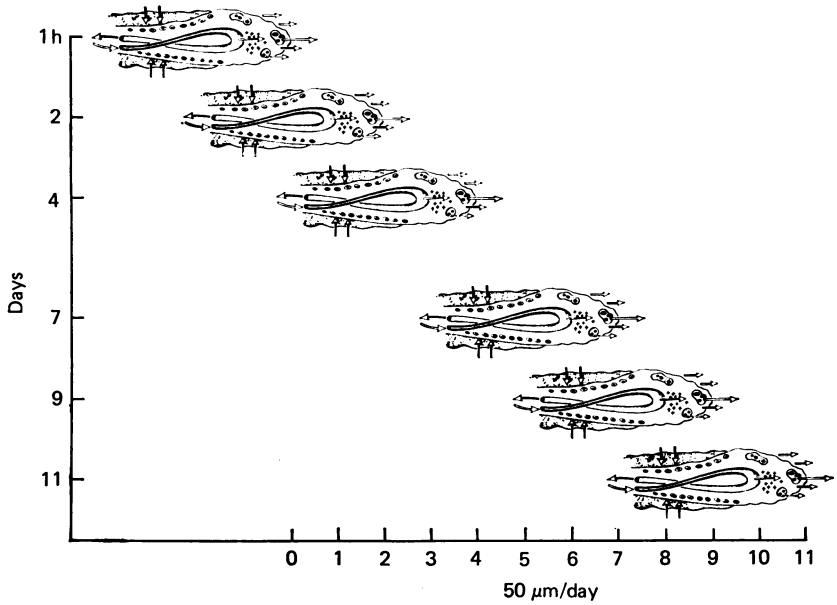


Fig. 1. Schematic demonstration of the preserved dynamic cytoarchitecture of an evolving Haversian system as it moves across the bone from the position at 1 hour to the situation at day 11 (adapted from Frost, 1963). The horizontal arrows in front of the Haversian system indicate the direction of the osteoclastic erosion and the system's advance, the vertical arrows the apposition of new bone by the osteoblasts on the wall of the eroded cavity.

MATERIAL AND METHODS

Rationale

Since thymidine becomes incorporated into the nuclei of cells rapidly synthesizing DNA, i.e. as they enter the premitotic phase, at one hour after the ^{3}HTdR injection such cells will be found labelled (i.e. containing grains) in radiographs. Subsequently, two kinds of labelled cells can be found. First, those which after the division differentiate into the functional cells. Such cells retain the label for the rest of their life span and through all their transformations, thus allowing us to follow the evolution of cells as they migrate. Second, the cells continuing to replicate themselves (the stem cells) which, with each division, dilute the label (i.e. rapidly proliferating cells lose the label fastest).

Serial longitudinal sections through the evolving secondary Haversian system permit the reconstruction of a three dimensional picture of its cytoarchitecture and the location of the various labelled and non-labelled cells within it. For instance, as the serial sections approach the bottom of the Haversian system, the cells which are seen transected sagittally, in the plane of the section through the middle of the system, may appear lying as it were *en face*, on the surface of the Haversian system as shown in Figures 3B–D. Furthermore, if the osteons in the biopsy material taken at various times after the injection of the ^{3}HTdR are so analysed, one can trace the evolution of cells (differentiation and migration) within the evolving secondary Haversian system as it moves through the bone.

Experimental design

Four young adult Beagle dogs weighing 10 kg were injected with tritiated thymidine (^{3}HTdR) 0·05 mCi/kg of body weight. Each dog underwent several rib biopsies (middle third of the rib, alternating the side and ascending the thoracic cage from the lowest ribs up) spaced at 1 hour (all dogs), 14 hours (one dog), 24 hours (two dogs), 2 (two dogs), 3 (two dogs), 4 (two dogs), 7 (two dogs), 9 (two dogs), and 11 (two dogs) days after the ^{3}HTdR injection. The fragments of ribs were fixed in 10 % neutral formalin, decalcified in EDTA and embedded in paraffin. Serial 5 μm longitudinal sections were cut in order to intercept the same evolving Haversian system in several longitudinal planes and prepared as autoradiographs according to the method of Kopriva & Leblond (1962). The sections were stained with eosine, dipped in liquid NTB 2 emulsion (Canadian Kodak Sales Ltd.), exposed for 5–14 weeks at 4 °C in dry air and developed in D19. They were counterstained with Harris' haematoxlin, dehydrated and mounted in Permount (Fisher Scientific Co. Ltd.). The serial sections were examined under bright light for the identification of cells with labelled nuclei and for grain counts.

RESULTS

Type, number and location of labelled cells at one hour after the thymidine injection (Table 1).

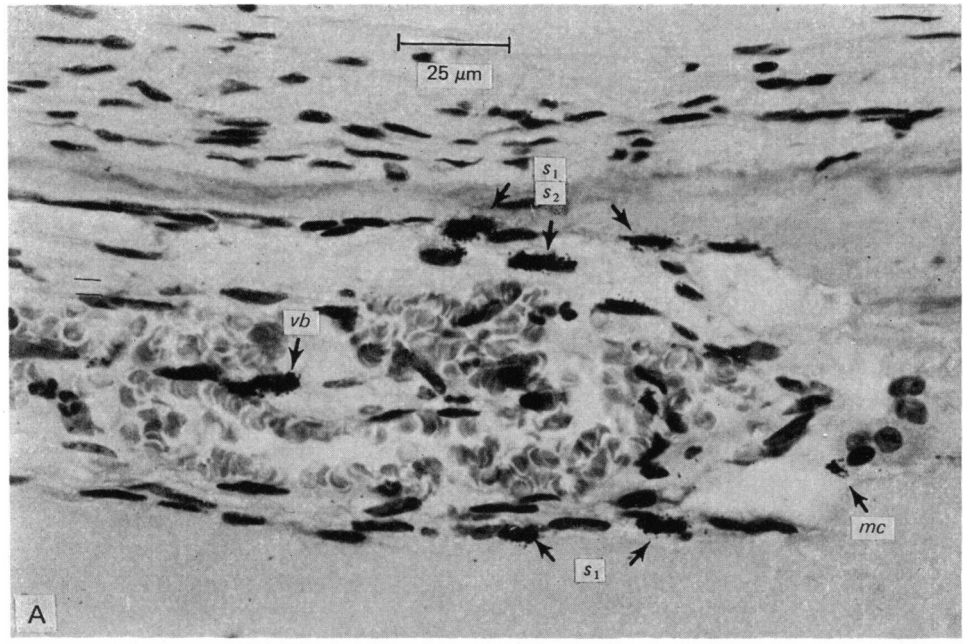
At one hour after the ^{3}HTdR injection (as shown in Figures 2A–C and Table 1) several types of labelled cells were seen: (1) large oval cells with pale cytoplasm and nuclei (mononuclear pre-osteoclasts, *mc*) in the vicinity of the osteoclasts (*c*); (2) spindle cells (osteoblast precursors, s_1) on the surface of the eroded cavity behind the osteoclasts, at the base of the cutting cone and in front of osteoblasts lining the most proximal part of the closing cone wall; (3) elongated cells with similarly oblong nuclei (stem cells, s_2) grouped in two or three loose layers located between the vascular axis and the osteoblasts (while the labelled cells of this kind were more numerous at the base of the cutting cone, they could be seen also along the whole closing cone); (4) cells of the vascular bud (*vb*) located behind the osteoclastic fronts; and (5) endothelial cells (*e*). Some of the labelled cells (*mc*, s_2, *e*) could also still be seen in a similar location 48 hours after ^{3}HTdR (Fig. 3A; Table 1).

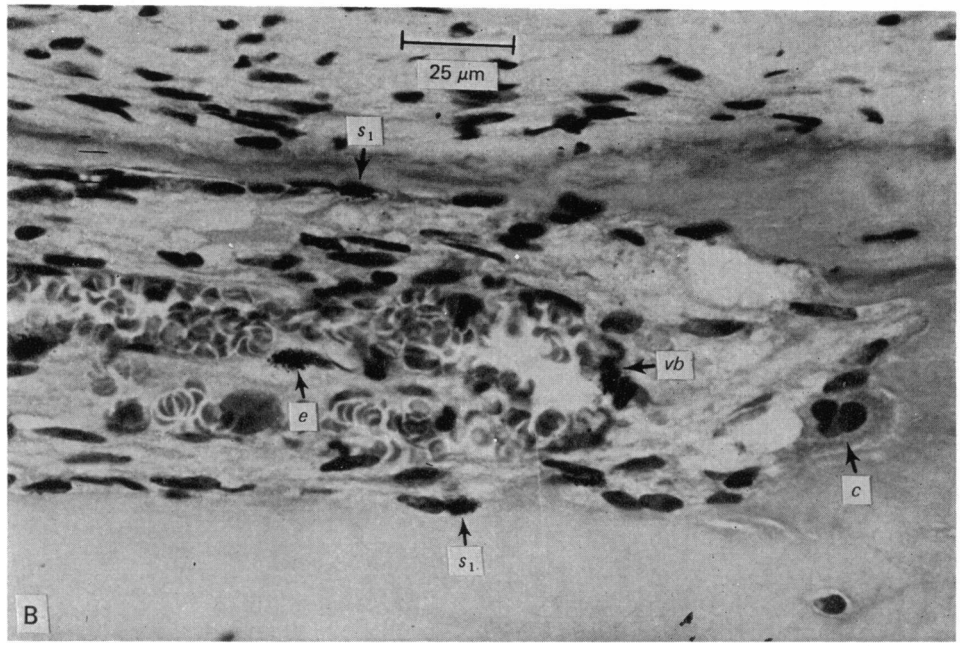
Type, number and location of labelled cells at various times after the injection of tritiated thymidine (Table 1).

The labelled osteoblasts (*b*), first noted at 14 hours and increasing in number up to 24 hours, were found in the most proximal part of the closing cone and then, with the passage of time, progressively in its more distant parts (Figs. 3A–D); labelled osteocytes were first seen at nine days (Fig. 5) in the distal part of the cutting cone.

During the time when the labelled osteoblasts appear, i.e. between the 14th and 24th hour, no labelled nuclei were found in the osteoclasts (Table 1). They were first seen at 24 hours, increasing moderately in number until the third day (Fig. 4) and decreasing slowly thereafter. Thus, osteoclasts with two or three labelled nuclei could be found up to the end of the period of observation when the cutting cone had moved about 550 μm from its original position (Table 1).

Labelled endothelial cells could be found along the capillary at a distance from capillary sinuses depending on the time of the biopsies.


25 μm
s_1
s_2
vb
mc
s_1
A


25 μm
s_1
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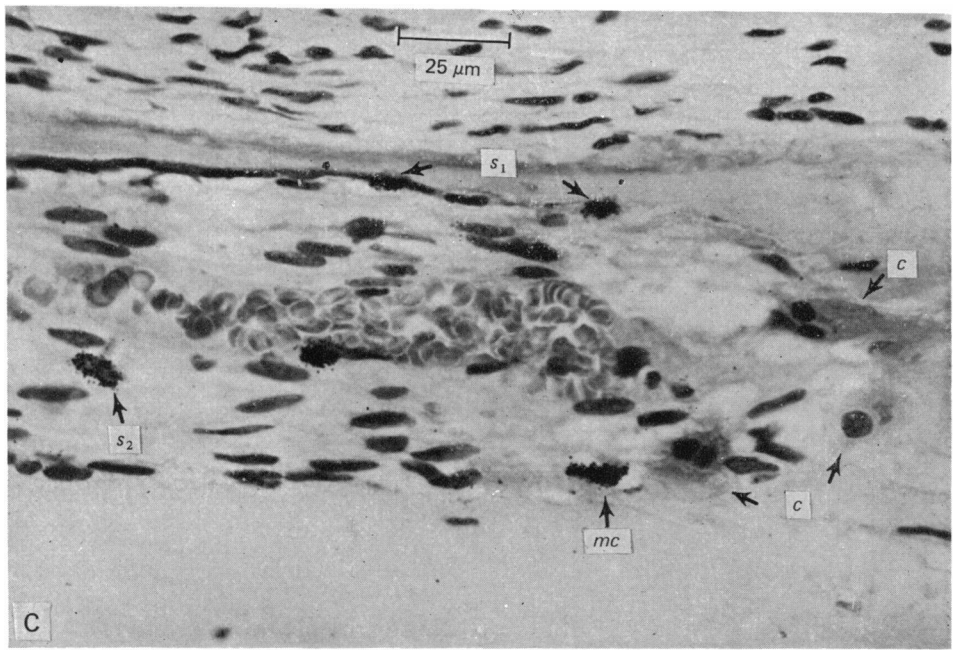


Fig. 2. Labelled cells in the evolving Haversian systems found one hour after ^{3}HTdR administration. Serial longitudinal sections (A, B, C) through the same Haversian system to show: large oval cells, presumably mononuclear preosteoclasts (*mc*), and located close to osteoclasts (*c*) with non-labelled nuclei; labelled spindle cells (s_1) at the base of the cutting cone and presumably the precursors of osteoblasts; elongated cells with large oval nuclei (s_2); heavily labelled cells in the vascular bud (*vb*) located behind the advancing osteoclast front; and endothelial cells (*e*).

The labelled elongated cells (s_2) could be seen at all times during the experiment and within the whole length of the closing cone.

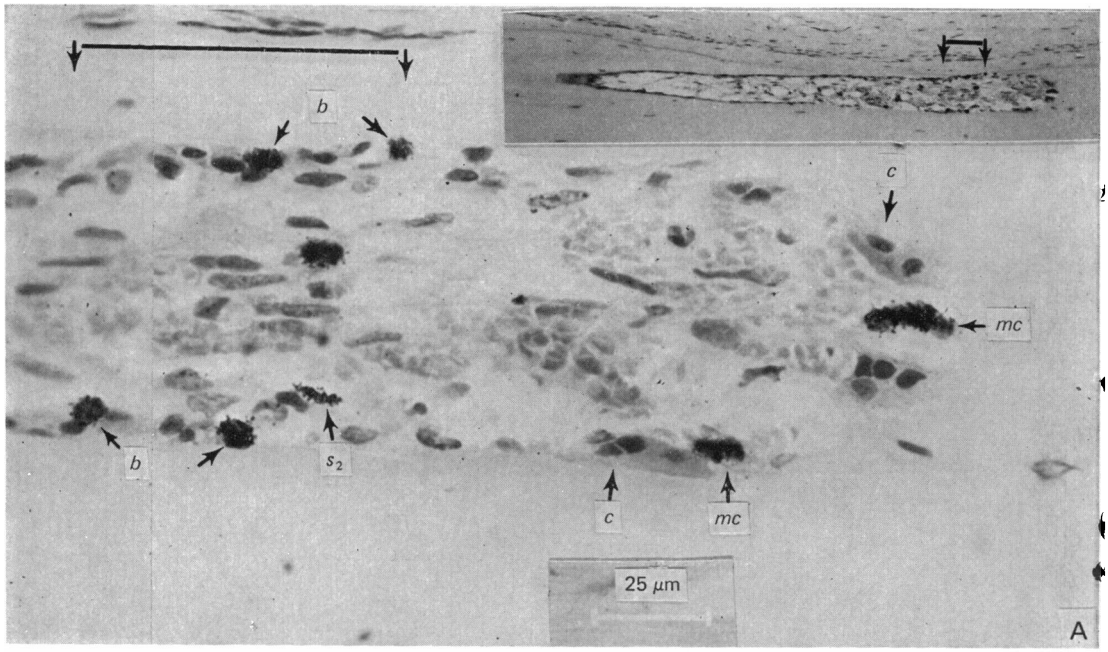
These observations are tabulated in Table 1 and presented schematically in Figure 6.

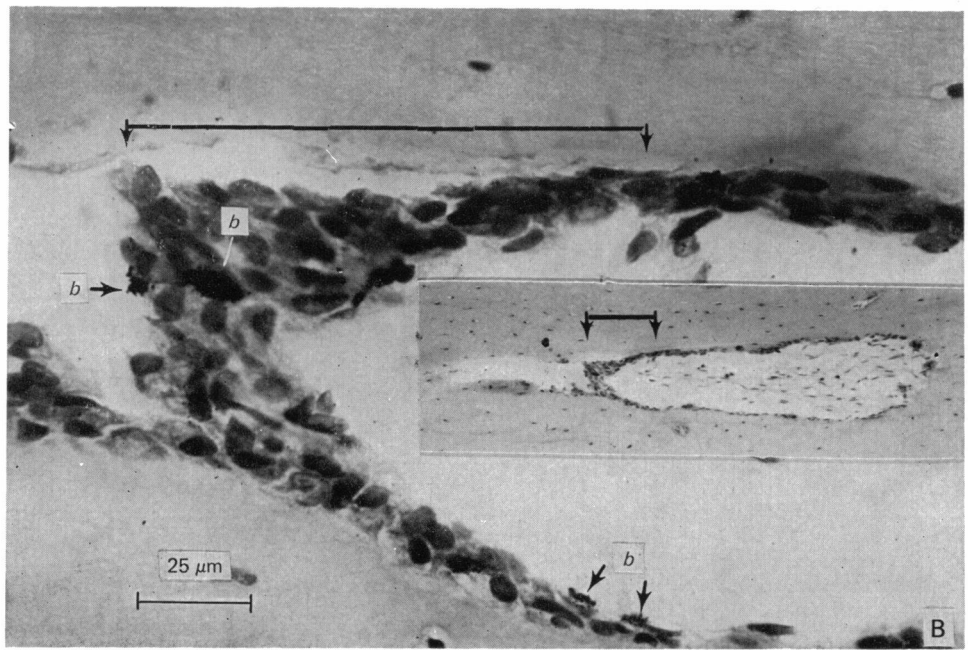
DISCUSSION

Tracking of ^{3}HTdR labelled cells within the evolving secondary Haversian systems allows us to draw some inferences regarding the origin, migration and fate of various functional cells as well as the relationships between the cell populations involved. Furthermore, the semi-quantitative estimate of the proportion of cells labelled within the population and their location within the evolving secondary Haversian system at various times after labelling gave us some indication as to the rates of various functional cells' recruitments.

As to the osteoclast population, the first few labelled nuclei were found at 24 hours, i.e. several hours after the first labelled osteoblasts had been seen. The number of labelled osteoclastic nuclei tended to increase slowly up to the seventh day (when few cells with two to three labelled nuclei could be seen), then declined slowly for the rest of the observation period.

Since at one hour after the ^{3}HTdR injection no labelled nuclei are found within the osteoclasts, their division within the constituted osteoclasts is unlikely. This finding is consistent with previous observations, suggesting that the osteoclasts originate by fusion of mononuclear cells (Hall, 1975; Hancox, 1972; Jee & Nolan, 1963; Tishman & Hay, 1962; Van Furth, 1970; Walker, 1973) and continue to acquire new nuclei,


b
c
mc
b
s_2
c
mc
25 μm
A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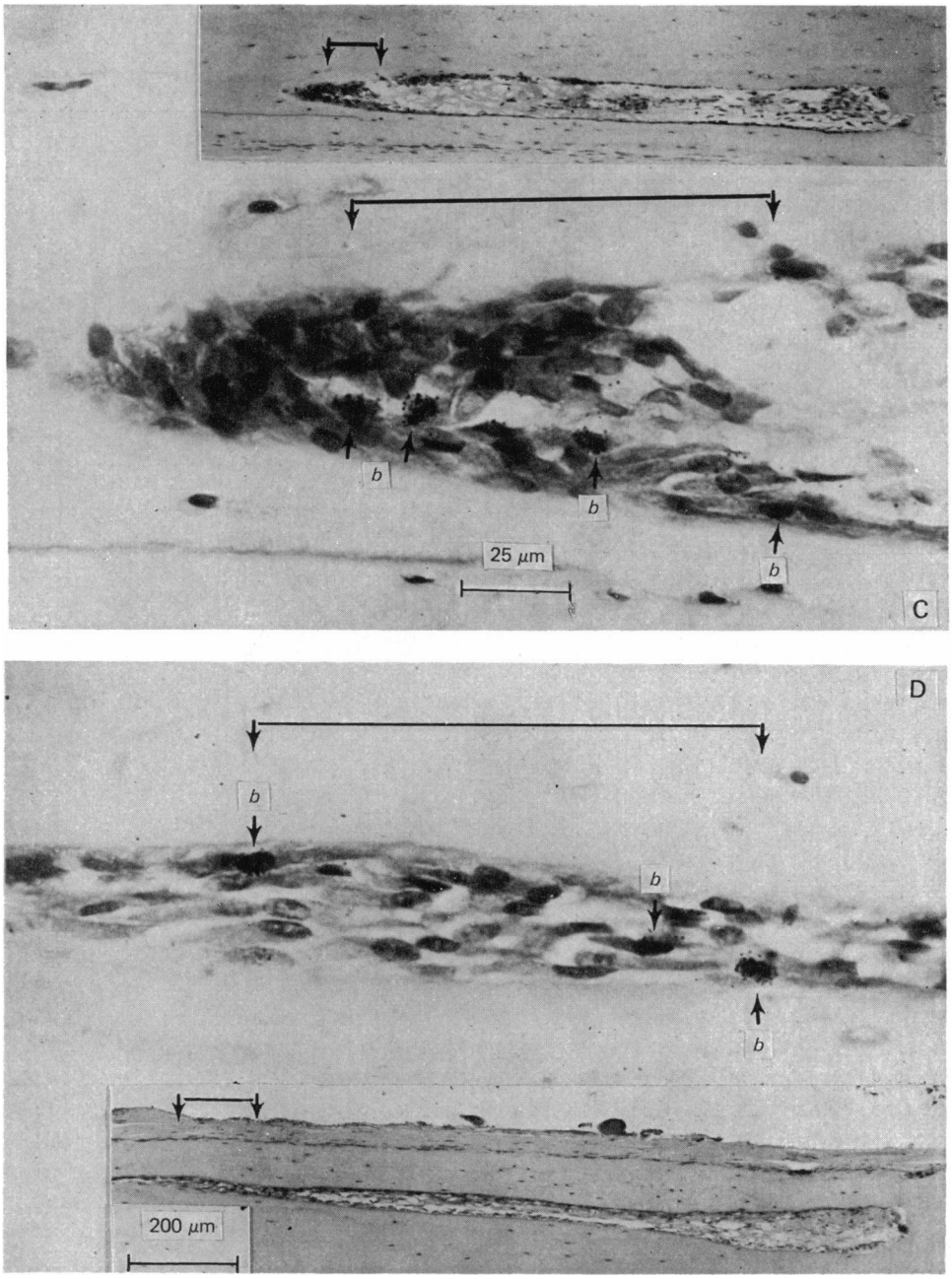

Fig. 3. Location of the zone with labelled osteoblasts in the closing cone of the evolving Haversian system as a function of time after the ^{3}HTdR administration. The whole length of the Haversian system under low magnification (small rectangle) contains the zone with labelled osteoblasts (indicated by the black line between the two arrows) which is shown also at eight times higher magnification (the length of the black line representing 200 μm and 25 μm respectively). Haversian system in the biopsy taken 48 hours (A), 3 days (B), four days (C) and nine days (D) after the ^{3}HTdR injection. The zone with labelled osteoblasts is left further behind as the time interval after the ^{3}HTdR injection increases and the Haversian system moves away from its position at that time. Because of the tangential cut, the surface of the osteoblast monolayer is seen in (B), (C) and (D), while in (A) the osteoblasts lining the wall of the Haversian system are transected by the plane of the section.

In the panel A the labelled cells in the vicinity of osteoclasts (*c*) are most probably the mononuclear osteoclasts' precursors (*mc*) which eventually fuse with the existing osteoclasts providing the labelled nuclei.

Table 1. *Type, location, number and appearance time of* ^{3}*HTdR labelled cells within the evolving secondary Haversian system*

Cell type	1 hour	14 hours	24 hours	48 hours	3 days	4 days	7 days	9 days	11 days
Large oval cells (*mc*) (mononuclear preclasts) (cutting cone)	X	X	X	X	X	X	occ	–	–
Osteoclasts (*c*) (cutting cone)	–	–	X	X	XX	XX	X	X	X
Spindle cells (s_1) (cutting cone)	XX	X	X	–	–	–	–	–	–
Osteoblasts (*b*) (closing cone)	occ	X	X	X	X	X	X	X	X
Osteocytes (*o*) (closing cone)	–	–	–	–	–	–	–	X	X
Elongated large cells (s_2) (cutting and closing cone)	XX	X	X	X	X	X	X	X	X
Vascular buds (*vb*)	X	X	–	–	–	–	–	–	–
Endothelial cells (*e*)	X	X	X	X	X	X	X	X	X

occ, occasional; XX, several; X, few

perhaps to replace the nuclei which they lose (Owen, 1963). While it is logical to assume that the heavily labelled cells at one hour found close to osteoclasts (*mc* cells in our study) may be the mononuclear precursors of osteoclasts, neither their lineage nor their origin, i.e. whether they arise locally or are brought by the bloodstream as speculated (Hall, 1975; Jee & Kimmel, 1977), could be decided on the basis of this study. The late appearance of few labelled nuclei within the osteoclasts and their slow increase in number suggests, however, that the osteoclast precursors (presumably *mc* cells) locally divide infrequently and that the turnover of osteoclast nuclei is slow. Consequently, the life span of osteoclasts must extend beyond the period of observation, i.e. 11 days. While the ultimate fate of osteoclasts also could not be determined, it is unlikely for the reasons to be discussed shortly, that they do differentiate and modulate, as has been postulated, into osteoblasts (Rasmussen & Bordier, 1974).

The fact that the first labelled osteoblasts appear behind the heavily labelled spindle cells (s_1 cells), first labelled at one hour and located at the base of the cutting cone, suggests strongly that these cells replicate themselves behind the advancing osteoclasts and differentiate to osteoblasts. This cellular advance is associated with the *longitudinal* apposition of bone at a speed which is that of the advancing osteoclastic front, i.e. some 40–50 μm a day (Jaworski & Lok, 1972). Assuming that these cells (both spindle cells and osteoblasts) measure some 10 μm in diameter, four to five new rows of spindle cells would be needed per day to cover the cavity surface left after the passage of osteoclasts. Thus, approximately four–five divisions of the spindle cells per 24 hours would be required to maintain the stem cell population and the recruitment of new osteoblasts. Since the proliferating spindle cells rapidly lose the label, few labelled osteoblasts appear after 24–48 hours. Consequently, as the osteoblasts usually do not divide further, the zone with the labelled osteoblasts is left increasingly behind with the passage of time and separated from the advancing cutting cone by the non-labelled generations of osteoblasts, as shown schematically in Figure 6. Assuming that the osteoblasts deposit a layer of bone 1·5 μm thick per day (Frost,

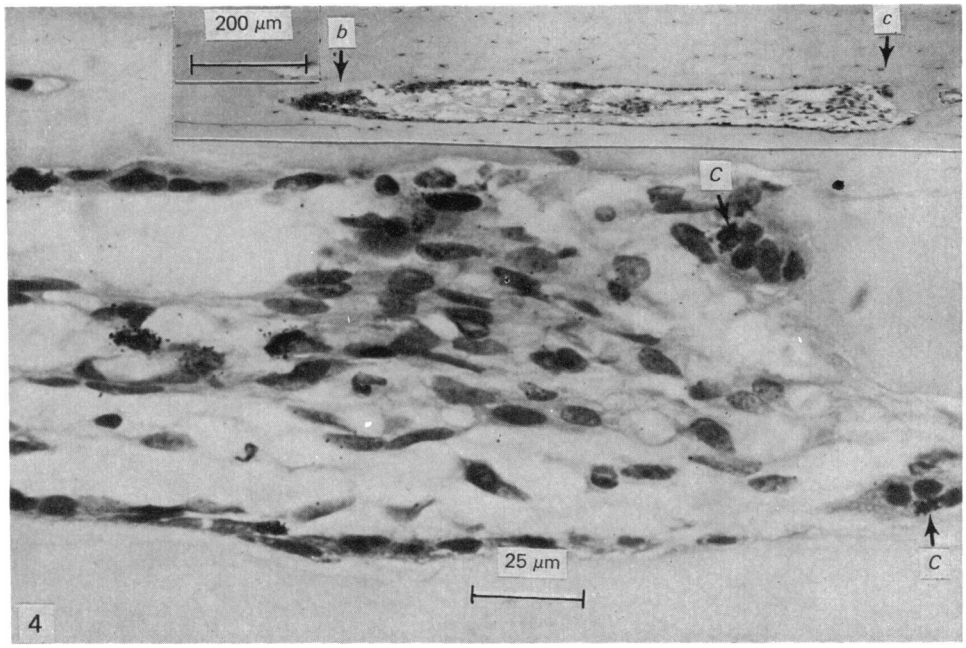

Fig. 4. A Haversian system from the biopsy taken 4 days after ^{3}HTdR injection, containing labelled nuclei within the osteoclasts (*c*); as shown in Fig. 3C, the same system contains labelled osteoblasts (*b*) in the closing cone.

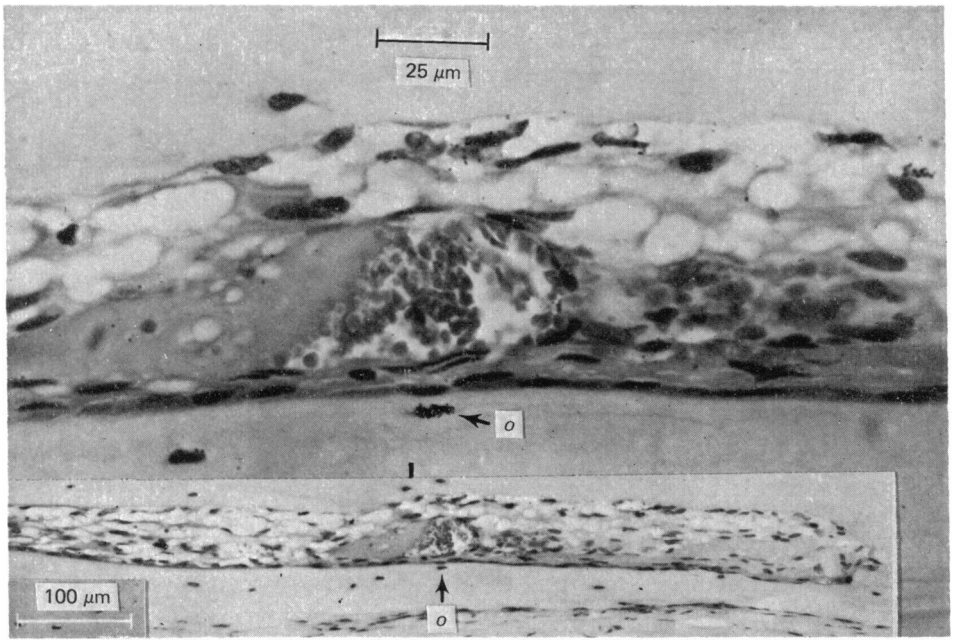

Fig. 5. Labelled osteocytes (*o*) are first noted nine days after the thymidine injection.

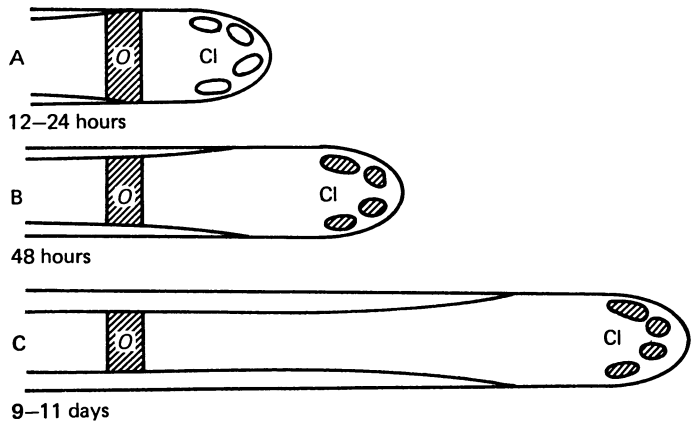

Fig. 6. Schematic representation of migration of labelled cells within the evolving secondary Haversian system: (A) at 14–24 hours, (B) at 48 hours and (C) at 9–11 days after the tritiated thymidine injection. At 14–24 hours (A) the zone with labelled osteoblasts (*O*) is located in the most proximal part of the closing clone (shaded area). At that time, there are as yet no labelled nuclei in the osteoclasts (Cl cutting cone). The first labelled nuclei are found in the osteoclasts at 48 hours (B), when the cutting cone (shaded area) has moved some 50–100 μm since the time of ^{3}HTdR. The zone of labelled osteoblasts is now seen left behind the advancing cutting cone. At 9–11 days (C), further separation occurred between the cutting cone with the labelled osteoclasts' nuclei (which has now advanced some 450–500 μm since the time of the ^{3}HTdR injection) and the zone with the originally labelled osteoblasts (for details see the text).

1963; Johnson, 1964), at 11 days, the zone with labelled (and now old) osteoblasts must have deposited *perpendicularly* to the wall of the eroded cavity a layer of new bone some 15–20 μm thick (hence the closing cone). Some of the osteoblasts from this zone are continuously lost as they become osteocytes embedded in the new bone. The period of observation of 11 days was not long enough to allow us to follow the zone with the labelled osteoblasts (no further than 500 μm within the closing cone) to determine for how long and how far it continues to deposit bone. It was, however, sufficiently long to suggest that the life span of functional osteoblasts in such systems is indeterminate, since it may range from some nine days to perhaps several weeks, when the few original osteoblasts become the endosteal cells lining the Haversian canal.

As to the spindle cells (s_1 cells) which have been assumed to give rise to osteoblasts, they may originate from large oblong cells (s_2 cells) first found heavily labelled at the base of the cutting cone, close to the capillaries and in the closing cone between the capillary axis and the osteoblast layer. These cells could thus represent stromal cells thought to give rise to endothelial cells of the capillaries or the osteogenic cells (Friedenstein, 1973; Lord, Testa & Hendry, 1975; Luk, Napajaroonsri & Simon, 1974; Owen, 1970).

The observations that the osteoblast precursors (s_1 cells) proliferate rapidly while the life span of osteoclasts is long and their nuclear turnover slow, provide a strong argument against the modulation of osteoclasts into osteoblasts (Rasmussen & Bordier, 1974). Thus, in order to satisfy the recruitment rate of new osteoblasts observed in this study, the osteoclastic nuclei would have not only to de-differentiate (presumably to the spindle s_1 cells) but also to acquire a capacity for rapid proliferation. The interpretation of data pertaining to cell kinetics in sites of lamellar bone remodelling reported here is therefore in agreement with the currently held theory

that the osteoclasts and osteoblasts derive from separate cell lineages, the osteogenic cells from the stromal cells and the osteoclasts from the pluripotential haematogenic cells (Friedenstein, 1973; Jee & Nolan, 1963; Jee & Kimmel, 1977; Lord *et al.* 1975; Tishman & Hay, 1962; Van Furth, 1970).

SUMMARY

A study of the origin, proliferation rate and migration of cells within the secondary evolving Haversian systems was undertaken in young adult Beagle dogs. Autoradiographs of serial longitudinal sections prepared from rib biopsies taken from one hour to eleven days after the injection of tritiated thymidine were subjected to semiquantitative analysis as to the time of appearance, number, location and transformation of various labelled cells.

Numerous labelled osteoblasts appeared early (at 14–24 hours) in the most proximal closing cone. With time, this zone was seen to have been left behind the advancing cutting cone and the successive generations of osteoblasts. The first labelled osteocytes were seen at nine days after injection, in the distal closing cone. Labelled nuclei within the osteoclasts were few and appeared late (none before 24 hours).

It is apparent that each self renewing cell population within these systems (i.e. osteoclasts, osteoblasts and endothelial cells) derives from its own immediate precursor and evolves at its own speed.

The mononuclear osteoclasts' precursors divide locally and infrequently and the turnover of osteoclastic nuclei appears to be slow; consequently their life span and that of the osteoclasts appears to be longer than the time of the observation, i.e. 11 days. The proliferation of osteoblasts' precursors and osteoblasts' recruitment is rapid. The life span of osteoblasts was found to be indeterminate; some osteoblasts may become osteocytes within a few days while others may continue to deposit bone for several weeks.

Since the recruitment of osteoclastic nuclei is slow while that of the osteoblasts is fast, it is unlikely that the osteoclasts in the sites of lamellar bone remodelling modulate into osteoblasts.

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