THYMIDINE-^{3}H ELECTRON MICROSCOPE RADIOAUTOGRAPHY OF OSTEOGENIC CELLS IN THE FETAL RAT

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ABSTRACT

The proximal tibial epiphyses of 18–21-day-old fetal rats have been studied by thymidine-^{3}H radioautography. The results reveal that the label is incorporated into two types of osteogenic cells: (*a*) a spindle cell type (A cells) with characteristics generally associated with matrix production, including an extensive development of the endoplasmic reticulum and the presence of large intracellular accumulations of a dense, finely granular material, morphologically identifiable as glycogen; and (*b*) a rounded cell type (B cells) with morphological features similar in degree and kind to those of the developing neutrophilic leukocyte, including an abundance of free ribosomes and mitochondria and a complex Golgi apparatus associated with dense specific granules, morphologically identifiable with primary lysosomes. These results, along with the occurrence of recognizable, labeled, immature, perivascular forms of both of these A and B type cells, lead to the conclusion that the specialization of osteogenic cells into osteoclasts and osteocytes may involve separate pathways of cytodifferentiation.

INTRODUCTION

Thymidine-^{3}H as a specific precursor of DNA has become a widely accepted marking tool for light microscope radioautographic study of the dynamics of cell proliferation (1–4). A major consideration which has contributed to the widespread use of thymidine-^{3}H is its specific incorporation into the newly synthesized DNA of any proliferating cell regardless of tissue, stage, or organism.

With this technique, much important information has been obtained on the proliferative rate and potential of the cellular components of bone (5–8). As a result of these studies, it is generally agreed that a population of proliferating mesenchymal cells constitutes a common progenitor pool from which all of the specialized bone cell types (i.e. osteoblast, osteocyte, and osteoclast) are derived. Due to the limitations of resolution obtainable by light microscope radioautography, these proliferating cells have not been precisely delimited in morphological terms. Thus, there is still controversy concerning criteria for identification of these cells. The issue has been further complicated by confusion as to the terminology to be employed in the designation of these proliferating cells and by conflicting opinions as to whether the range of their inherent developmental capacity includes other connective tissue cell types as well as the specialized bone cells.

With the adaptation of thymidine-^{3}H techniques for electron microscopy (9–11), critical evidence has been forthcoming on the relation-

ship of proliferation and cytodifferentiation in a number of vertebrate cell types including the neutrophilic leukocyte (12). As yet, however, there is little direct information on the fine structure of proliferating cells in bone tissue.

The purpose of this communication is to report preliminary findings on the fine structure of proliferating osteogenic cells as revealed by thymidine-^{3}H electron microscope radioautography and to consider these findings in light of some of the current terminologies and cytogenetic theories.

MATERIALS AND METHODS

Pregnant Long-Evans rats (18–21 days gestation) weighing from 350 to 400 g were injected intraperitoneally with an aqueous solution of thymidine-^{3}H (New England Nuclear Corporation, Boston, Mass; specific activity, 6.7 c/mmole) at a dosage level of 10 μc/g body weight.

1 hr after injection, the mothers were anesthetized with phenobarbital and the fetuses were exposed individually, the placental circulation being left intact. The proximal tibial epiphyses were excised from the fetuses and fixed for 10 min in cold phosphate-buffered formaldehyde (13), after which they were postfixed in cold phosphate-buffered osmium tetroxide (14) for 30 min to 1 hr. Subsequent to dehydration in a graded series of alcohol, the specimens were embedded in Epon by Luft's method (15). Sections were cut on a Porter-Blum microtome with a diamond knife.

For electron microscope radioautography, thin sections (in the range of 1000 A thickness) were mounted on copper grids with carbonized Parlodion supporting films and coated with Ilford L-4 emulsion by the loop method (9). Exposure was for 4–6 wk at 4°C. Radioautographs were developed in a 1:2 dilution of Dektol at 20°C for 4 min, fixed in Kodak fixer for 2 min, washed, then stained with uranyl acetate and/or lead citrate (16). The radioautographs were examined in an RCA EMU 3E microscope the qualitative uptake of thymidine-^{3}H in the tissue as indicated by the presence of silver grains over the nuclei of the cells. The observations reported here are limited to labeled cells in the primary spongiosum characteristically found on the surface of the developing bone trabeculae or along the capillaries in the immediate vicinity.

While these particular labeled cells are selectively considered in this report, they do not constitute the only labeled cell types observable within the primary spongiosum. Other labeled cell types which have been observed in the same radioautographs of the primary sponsiosum include: capillary endothelial cells, hemopoietic cells, and histiocytic cells. These labeled cells, along with those described in the present report, will be considered in a more extensive thymidine-^{3}H electron microscope radioautographic study which is currently underway.

RESULTS

1 hr after the administration of thymidine-^{3}H, the label appears in a relatively large proportion of the uninuclear cells that are characteristically associated with the developing bone trabeculae or the nearby capillaries (Fig. 1).

On the basis of their shape, size, and appearance, these labeled cells are divisible into two types, which are arbitrarily designated here as *A* and B cells. In addition, within each of these two cell categories, two subtypes are recognizable on the basis of their relative topographical distribution and degree of cytoplasmic organization: (*a*) small, immature perivascular A and B cells; and (*b*) larger endosteal A and B cells exhibiting a comparative high degree of cytoplasmic complexity.

A Cells

PERIVASCULAR SUBTYPE: The A cells are represented by elongated, outstretched elements (Fig. 1), which in profile are typically spindle shaped (Fig. 2). These cells range roughly from 5 × 10 μ to 15 × 40 μ in size. The smallest cells are characteristically localized in pericapillary sites where they are usually distributed singly in close topographical relationship to the capillary endothelium. This disposition of the cells is exemplified in Fig. 2. They are typically seen to be separated from the capillary endothelium by a demonstrable extracellular space approximately 1–2 μ in width.

In general appearance, the small A cells present a strikingly immature aspect (Figs. 1, 2). The nucleo-cytoplasmic ratio is high, a large, oval nucleus occupying over ¾ of the total cell volume. Characteristically, the chromatin of the nucleus is loosely and evenly distributed. Sometimes, a single small nucleolus may be observed within the nucleus. The cytoplasm of these cells is represented by a very thin rim surrounding the nucleus. Aside from a few isolated fragments of rough surfaced lamellar components of the endoplasmic reticulum, the cytoplasm exhibits a marked paucity of formed organelles. The cell surface is bounded by a simple plasmalemma without any demonstrable basement membrane.

ENDOSTEAL SUBTYPE: The small perivas-

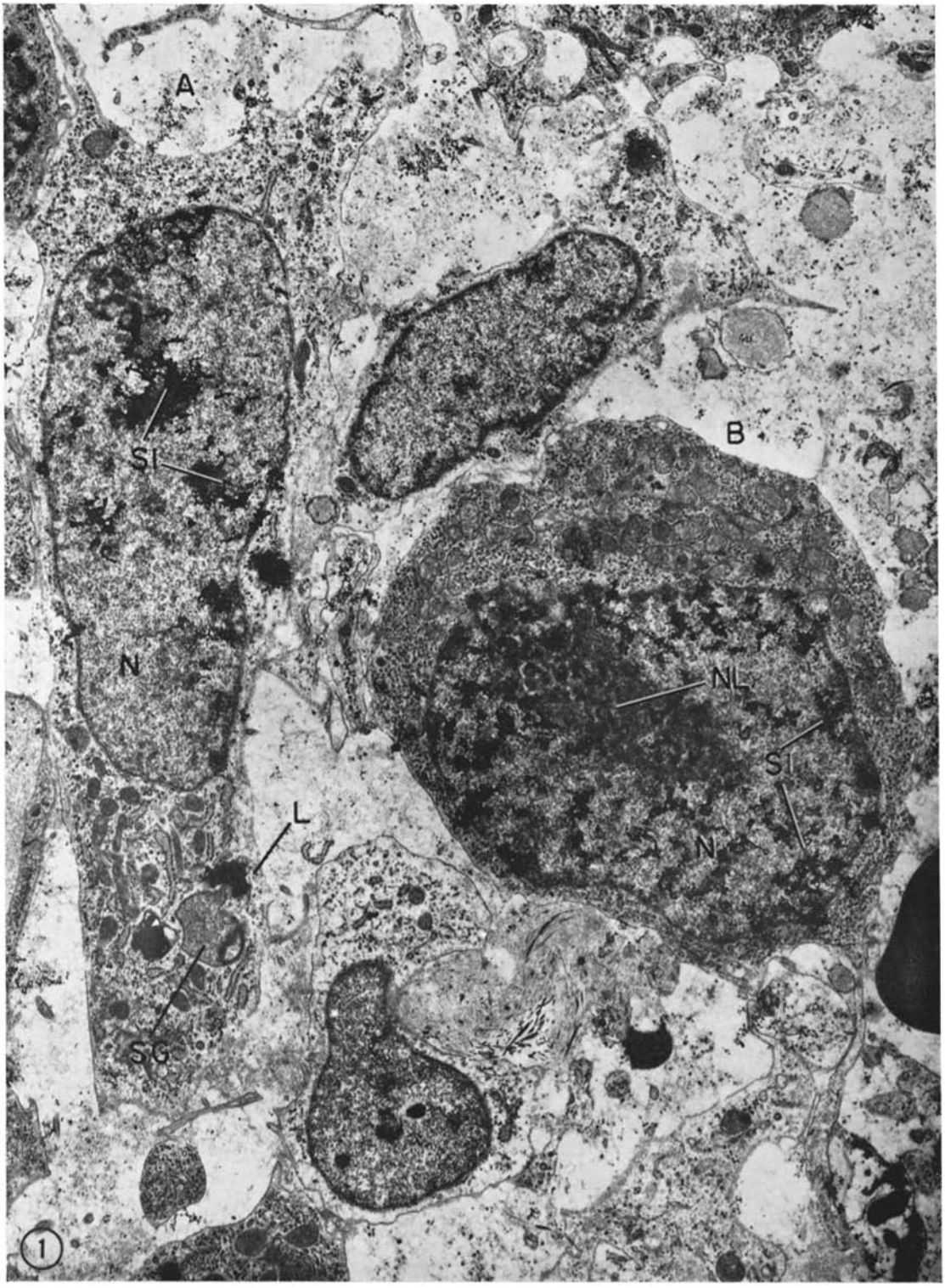
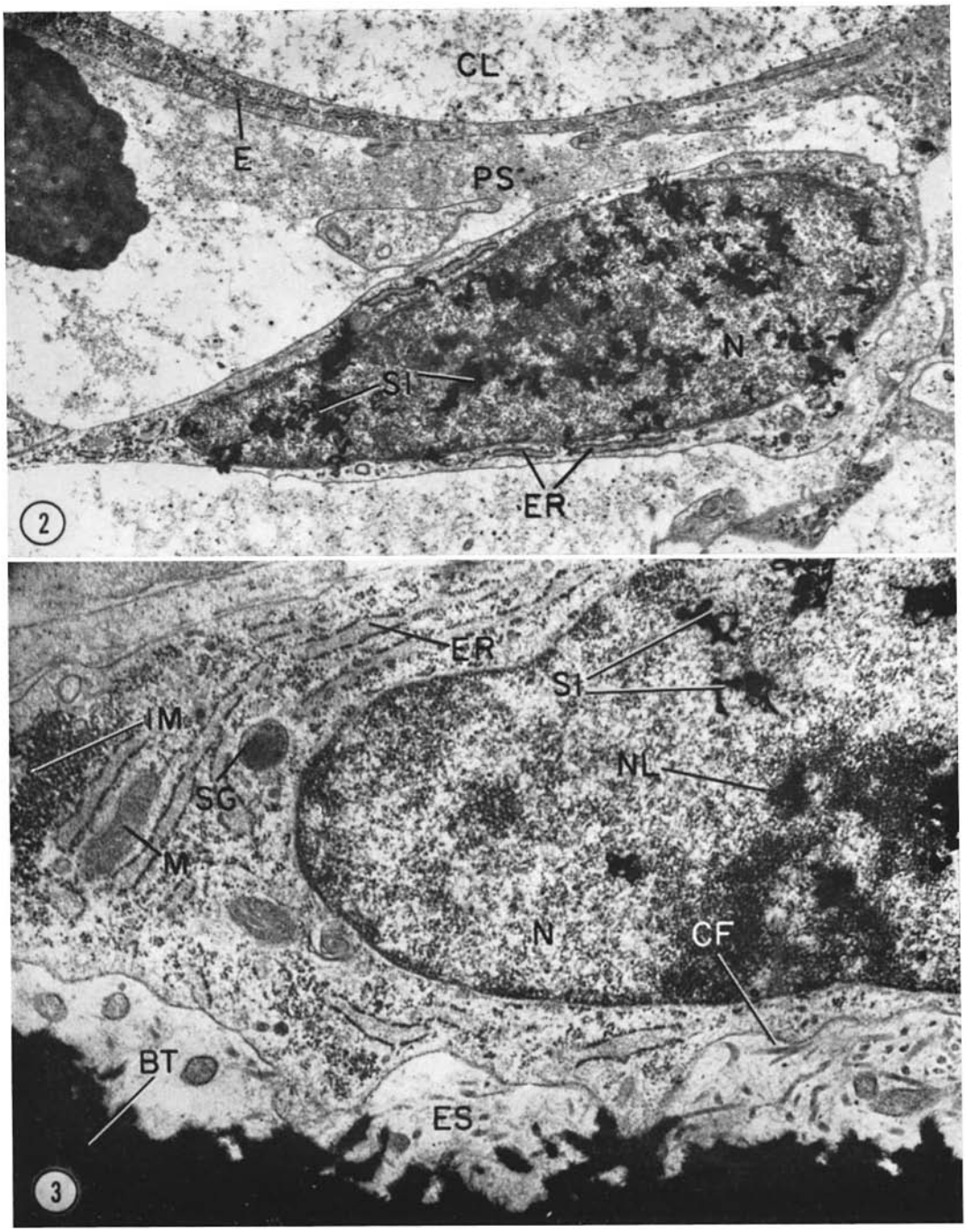


FIGURE 1 Survey radioautograph of an intercapillary space in the primary spongiosum to illustrate the general appearance and distribution of the labeled cells. They are seen to consist of a fusiform cell type (*A*) and a rounded cell type (*B*). Note the marked, over-all density and coarse granularity of the cytoplasm of the B cell. *N*, nucleus; *NL*, nucleolus; *Sl*, silver grains; *L*, lipid droplet; *SG*, specific granule. × 15,000.



FIGURES 2 and 3 These radioautographs illustrate the marked differences between the perivascular (Fig. 2) and the endosteal (Fig. 3) representatives of the A cells in terms of size, nucleocytoplasmic ratio, and degree of cytoplasmic organization. In the endosteal A cell (Fig. 3), note the relative conspicuous development of the endoplasmic reticulum (*ER*), the granular inclusion material (*IM*), and the collagen fibrils (*CF*) in the extracellular space (*ES*) between the cell surface and the related bone trabeculum (*BT*). *N*, nucleus; *NL*, nucleolus; *Sl*, silver grains; *M*, mitochondria; *SG*, specific granule; *E*, endothelium, *CL*, capillary lumen; *PS*, pericapillary space. × 18,000.

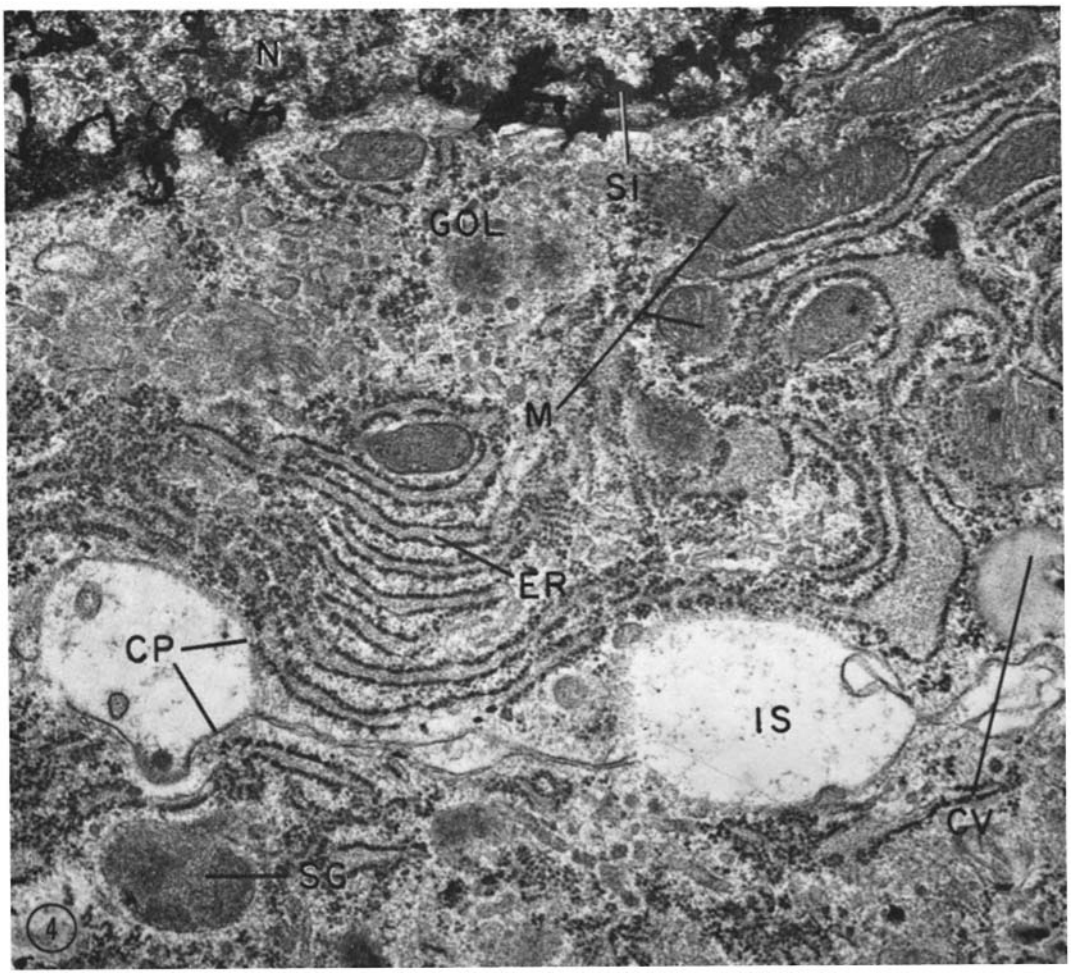


FIGURE 4 Cytoplasm and intercellular junction of adjacent endosteal A cells. Points of contact between the two cells are seen to be established by short, cytoplasmic processes (*CP*). *N*, nucleus; *Sl*, silver grains; *GOL*, Golgi apparatus; *M*, mitochondria; *ER*, endoplasmic reticulum; *SG*, specific granule; *CV*, cytoplasmic vesicle; *IS*, intercellular space. × 26,000.

cular A cells are linked by a series of transitional forms to larger, more complex A cells. These larger A cells are usually found distributed singly, or in small groups, along the surface of the developing bone trabeculae (Fig. 3). Between the bone matrix and the opposed cell surface is an extracellular space, approximately 2–5 μ, in width, within which a varying accumulation of randomly oriented collagen fibrils is characteristically encountered.

The cytoplasm of the large A cells is distinguished by an extensive development of the endoplasmic reticulum. This organelle appears in the form of a system of parallel lamellae with numerous attached ribosomes (Figs. 3–5). Dilations of the lamellae are often filled with a homogeneous material of moderate electron opacity, and similar material is also seen to be contained in the unexpanded portions of the lamellae (Fig. 4).

By comparison with the endoplasmic reticulum, the Golgi apparatus is poorly developed (Fig. 4). It is usually distinctly recognizable, however, as a single localized perinuclear concentration of isolated smooth-surfaced lamellae and vacuoles. The mitochondria are well represented, being randomly disposed in the cytoplasmic interstices

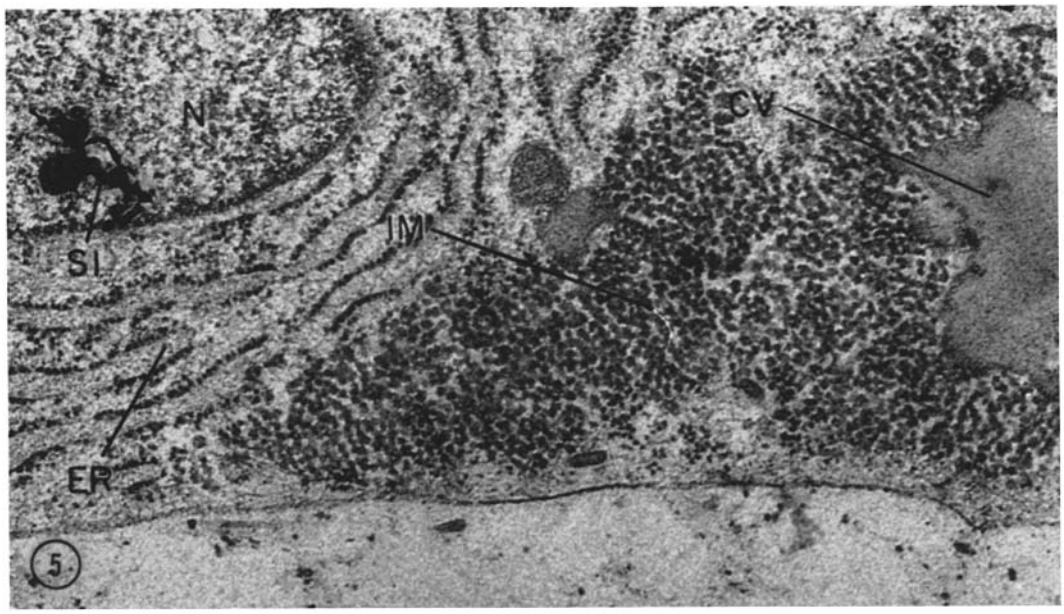


FIGURE 5 A portion of the peripheral cytoplasm of an endosteal A cell to illustrate details of the granular inclusion material (*IM*) noted in Fig. 3. *N*, nucleus; *Sl*, silver grains; *ER*, endoplasmic reticulum; *CV*, cytoplasmic vesicle. × 55,000.

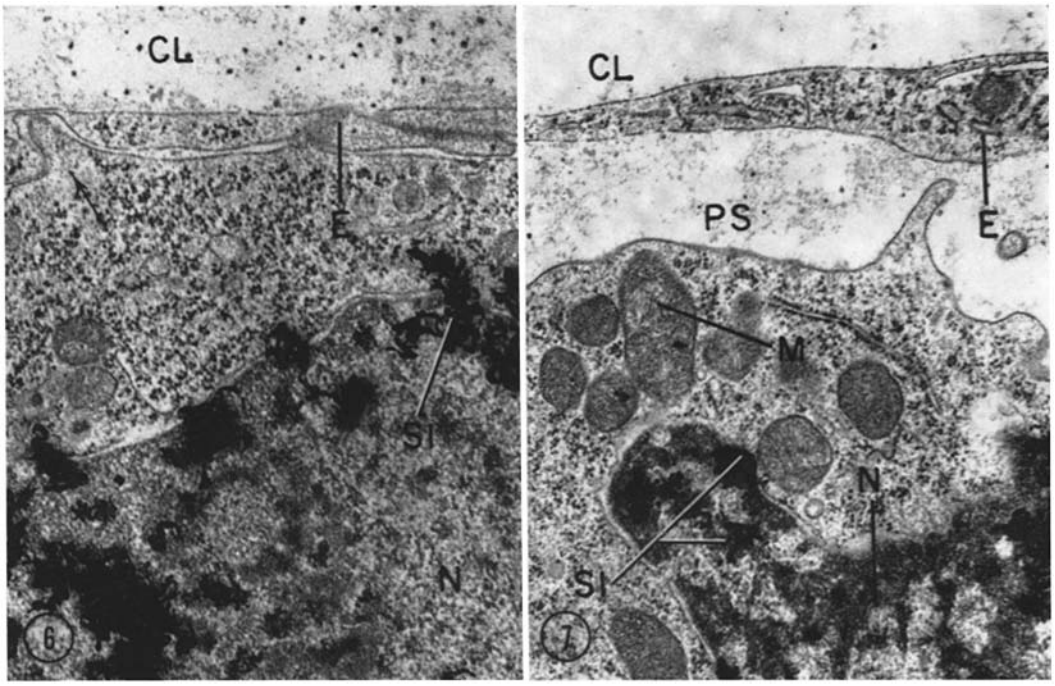
between the lamellae of the endoplasmic reticulum (Fig. 4). They generally appear as dense, short, rod-shaped profiles with well developed internal cristiform organization. Frequently, one also encounters a variable number of lipid droplets (Fig. 1) and one or two large granules of varying density (Figs. 1, 3, 4) within the A cells at any particular stage of cytodifferentiation.

Aside from these features, a notable characteristic of the large A cells is the accumulation of massive intracellular deposits in the peripheral cytoplasm (Figs. 3, 5). These deposits are composed of a finely granular material which exhibits a high affinity for lead stains. The component particles of this material are more or less uniform in size (200–250 A in diameter) and are closely packed. Sometimes, within the midst of the particles, one may observe one or more large membrane-bounded vesicles filled with a homogeneous material of moderate electron opacity (Fig. 5). Similar vesicles may also be encountered in varying numbers along the inner surface of the plasma membrane (Fig. 4).

In addition to showing these internal differences as compared to perivascular forms, the endosteal A cells also reveal marked differences in contour owing to the presence of larger numbers of cytoplasmic processes (Figs. 1, 3, 4). At the cell surface adjacent to bone, the cytoplasmic processes are often seen to terminate directly upon the calcified surface of bone (Fig. 3). In other regions of the cell, the cytoplasmic processes may establish contact with those of neighboring A cells to form an intercellular lattice (Fig. 4). At the point of contact, the surfaces of the related cells remain discrete, with no demonstrable signs of cytoplasmic continuity.

B Cells

PERIVASCULAR SUBTYPE: The B cells typically present a rounded profile in sections (Fig. 1). The diameter of these cells varies markedly, ranging from approximately 25–75 μ. The smallest, most immature forms of the B cells, like their A counterparts, exhibit a pericapillary disposition. Among the different B cells, however, there is a marked variation in their relative proximity to the endothelial wall. At one extreme, the cells may be closely applied to the endothelial wall, often interdigitating with it at surface points (Fig. 6). At the other extreme (Fig. 7), the B cells may be more remotely removed from the



FIGURES 6 and 7 These radioautographs illustrate the relative proximity of different immature B cells to the invading capillaries (*CL*). In Fig. 6, note the point of interlocking (arrow) between the B cell and the capillary endothelium (*E*). *N*, nucleus; *Sl*, silver grains; *M*, mitochondria; *PS*, pericapillary space. Fig. 6, × 18,000; Fig. 7, × 25,000.

endothelial wall, being separated from it by a fairly wide interspace (approximately 0.5–2 μ in width) comparable to that observed for the perivascular A cells (Fig. 2).

In common with the perivascular A cells, the perivascular B cells also exhibit a high nucleocytoplasmic ratio. The nucleus of the individual cells is eccentrically placed and generally rounded in shape with an irregular undulating contour (Fig. 1). The chromatin is more abundant and unevenly dispersed than in comparable A cells. It is primarily represented by large aggregated masses located near the nuclear membrane. In addition, one or more large nucleoli may be observed within the nucleus.

Despite their general immature appearance, the perivascular B cells share several basic features of cytoplasmic organization with the larger, more complex endosteal B cells (Figs. 8, 9). One of these basic features is the presence of an abundant distribution of free ribosomes throughout the cytoplasm (Figs. 6, 7). Thus, aside from their difference in shape, even upon low-power survey (Fig. 1), these cells are easily distinguishable from their A counterparts (Figs. 1, 2) by their marked over-all density.

Another basic cytoplasmic feature which the small perivascular B cells share in common with the larger representatives is the presence of conspicuous numbers of small mitochondria, which impart a coarse granularity to the cytoplasm at low magnification (Figs. 1, 7). The mitochondria are typically globular in shape, although rod-shaped forms are also often observed. Except for one or two occasional intramitochondrial granules, the matrix of the mitochondria is homogeneous with an electron opacity approaching that of the cytoplasmic matrix. The internal cristae of the mitochondria are fairly sparse in numbers and irregularly spaced.

ENDOSTEAL SUBTYPE: Representatives of the larger B cells (Fig. 8) are constantly found scattered at random points along the surface of the developing bone trabeculae. These cells are usually separated from the bone by a demonstrable interspace. Sometimes, however, the

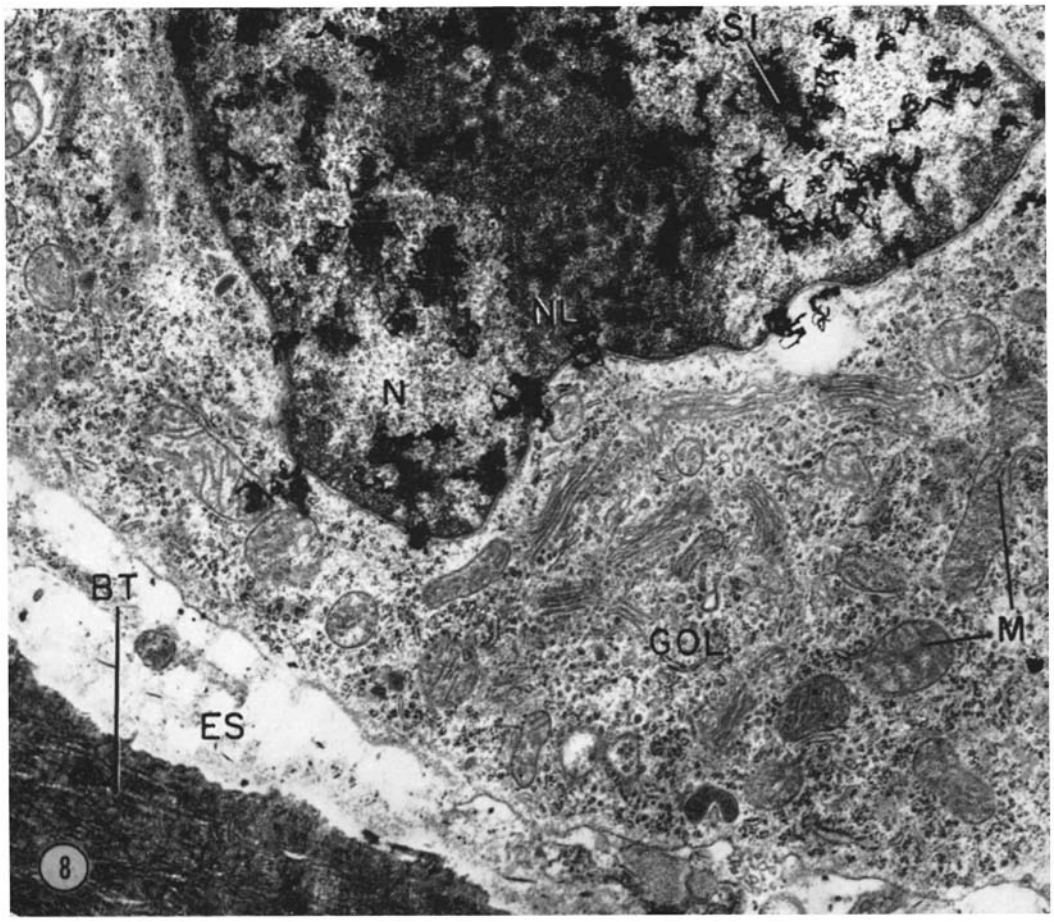


FIGURE 8 Survey view of an endosteal B cell. Note the irregularity in contour of the nucleus (*N*) and the uneven distribution of chromatin. In the cytoplasm, note the extensive Golgi apparatus (*GOL*), and the relative abundance of mitochondria (*M*) and free ribosomes. *NL*, nucleolus; *Sl*, silver grains; *BT*, bone trabeculum; *ES*, extracellular space. × 18,000.

opposing cell surface may directly abut upon the bone surface throughout much of its extent.

The nucleus of the large B cells varies in shape. In the large majority of the cells, the nucleus is spherical but its surface is provided with numerous deep fissures which impart a general lobated appearance. In some of the other B cells, the nucleus may assume a horseshoe shape.

In addition to having basic cytoplasmic features which they share in common with the perivascular forms, the endosteal B cells are further characterized by an extensively developed Golgi apparatus (Figs. 8, 9). This organelle is represented by one or more perinuclear units, each of which is composed of multiple arrays of stacked lamellae and associated vacuoles. In its highest state of complexity, the Golgi apparatus is seen to be associated with specific granules in all apparent stages of formation (Fig. 10). These granules appear as rounded or oval membrane-bounded bodies about 0.5–1.0 μ in diameter and are filled to varying degrees with a dense homogeneous material. They are found typically within the midst of or at the periphery of the Golgi apparatus. However, completely formed granules of similar size may frequently be observed at random sites in other cytoplasmic regions.

As in the case of their A counterparts, a series of transitional forms may be observed between

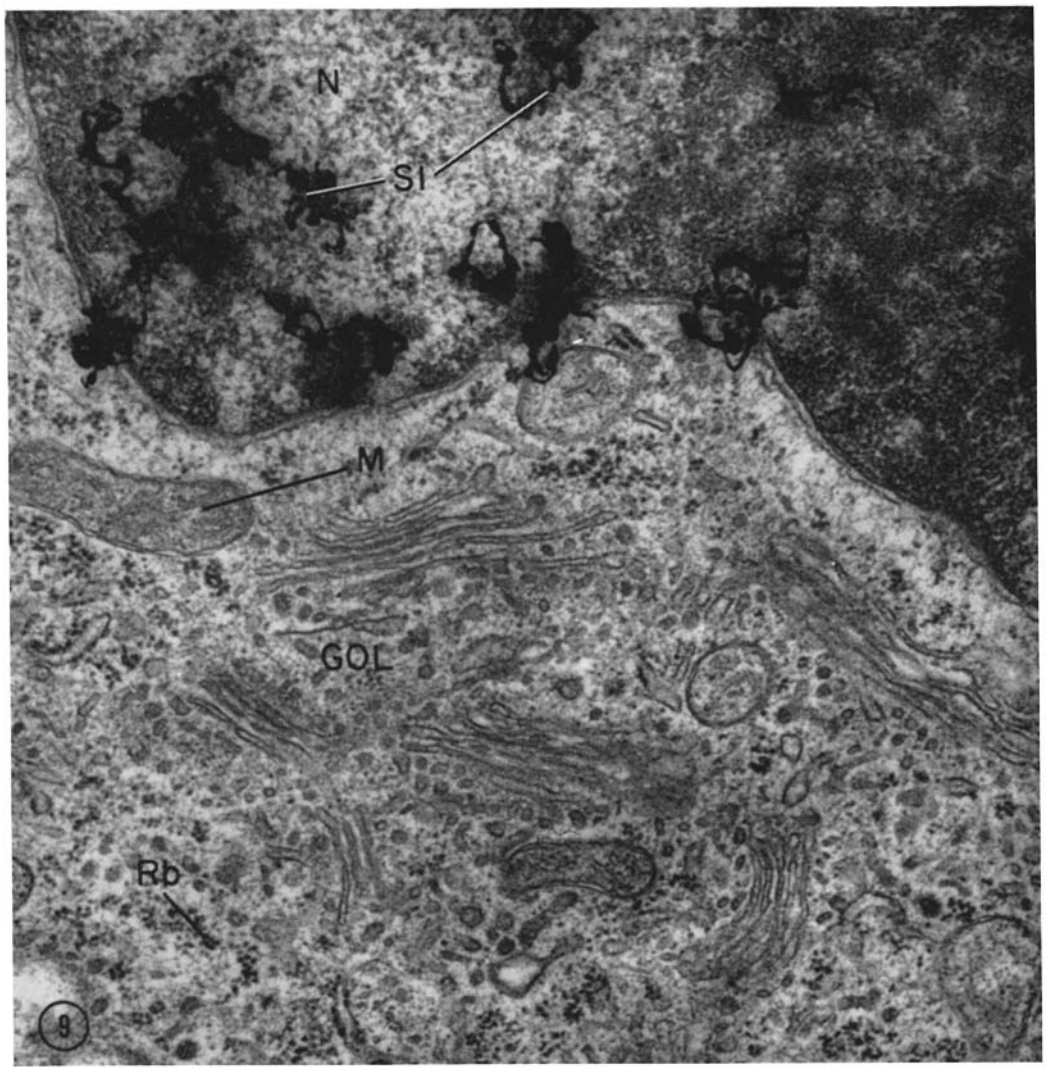


FIGURE 9 Details of cytoplasmic organization in the endosteal B cell illustrated in Fig. 8. Note the multiple units of stacked lamellae of the Golgi apparatus (*GOL*). *M*, mitochondria; *N*, nucleus; *Sl*, silver grains; *Rb*, ribosomes. × 45,000.

these endosteal B cells and the perivascular B cells. It should be noted at this point, however, that recognizable transitional forms between the A and B subtypes were not encountered.

DISCUSSION

Classification of the Labeled Cells

In the past, primarily on the basis of gross morphological criteria, the proliferating cells at sites of active osteogenesis have been designated by a number of general descriptive terms, including spindle cells, reticulum cells (17), and mesenchymal cells (18). The two most widely used current terminologies, however, are based on criteria of osteogenic developmental potency. According to one school of thought, all of the proliferating osteogenic cells undergo primary specialization into osteoblasts which then may undergo further specialization into either osteo-

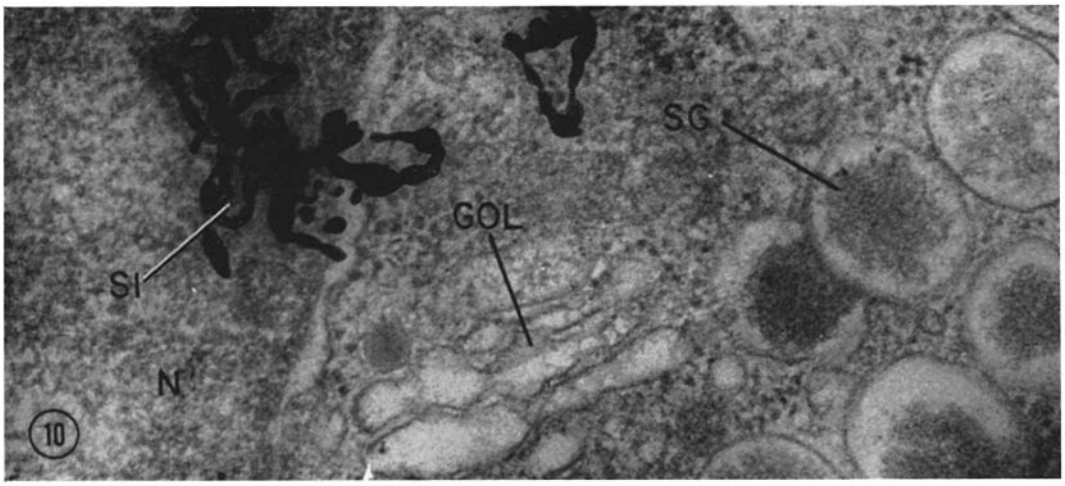


FIGURE 10 Relatively small field of perinuclear cytoplasm of an endosteal B cell in a more advanced state of cytodifferentiation to illustrate the relationship between the Golgi apparatus (*GOL*) and specific granules (*SG*). *N*, nucleus; *SI*, silver grains. × 95,000.

cytes or osteoclasts (19). Thus, to connote this concept, all the osteogenic cells have been inclusively designated under the generic term "preosteoblast" (19). This taxonomy has been supported by numerous light microscope thymidine-^{3}H studies, notable among which are those of Tonna (6) and Owen (8). Young (7), on the contrary, has obtained light microscope radioautographic results which led him to conclude that the osteoclasts as well as the osteoblasts represent primary derivatives of the proliferating precursor cells. In order to embrace both pathways of specialization, Young has, therefore, proposed to designate the precursor cells under the broader generic category of "osteoprogenitor cells." He specifically includes in this classification the cells on or near the surface of bone or calcified cartilage which have inconspicuous cytoplasm and pale vesicular oval or fusiform nuclei.

The present electron microscope radioautographic results would seem to be more compatible with Young's concept. These results have demonstrated that the labeled cells characteristically associated with the developing bone trabeculae are composed of fusiform A cells and rounded B cells, which are delineated on the basis of their features of cytoplasmic differentiation as well as their external form. The developing A cells are seen to possess the morphological criteria generally associated with matrix-formation in connective tissue cells. Included among these features is an extensive development of the endoplasmic reticulum indicative of the intracellular synthesis and transport of the proteins utilized in collagen production (20). Correlated with this finding are overt signs of the secretory process manifested by the accumulation of formed collagen fibrils within the extracellular space in the immediate vicinity of the A cells.

Another electron microscope criterion of matrix-producing cells which is also demonstrable in the A cells is the occurrence of massive intracellular deposits of a material with the fine-structural and lead-staining characteristics of glycogen (21). Among the recognized matrix-producing cells, such deposits have been shown to be particularly exemplified in the chondrogenic cell types (i.e. chondroblasts and chondrocytes), where they have been strongly implicated in the intracellular production of the acid polysaccharide components of the matrix (22).

On the basis of these collective features of the A cells and their topographical relationship to the developing bone, these labeled cells are recognizable as the counterparts of the developing osteoblasts described in the same specimen material by conventional electron microscopy (23). Similar cells have also been shown in in vitro electron microscope studies to be directly transi-

tional with the definitive osteoblasts in the avian embryo (20). Thus, in a restricted morphological connotation the term "pre-osteoblast" (19) would seem to be applicable to the A cell lineage.

In contrast to the A cells, the developing B cells more closely resemble the neutrophilic leukocyte (12, 24, 25) than the osteoblast. In common with the neutrophilic leukocyte, the B cells are characterized by an abundance of free ribosomes, mitochondria, and an extensive Golgi apparatus. In its higher stages of complexity, the Golgi apparatus is associated with incipient forms of specific granules. These granules are very similar in size and appearance to those of the neutrophilic leukocyte, which are known to represent primary or storage forms of hydrolytic enzymes involved in intracellular digestive mechanisms (26). In this regard, it is interesting to note that similar granules have also been observed in the immature and definitive osteoclasts described in electron microscope studies on unlabeled fetal rat epiphyses (23, 27).

In sum, these considerations permit us tentatively to reserve the term "pre-osteoclast" for the second labeled cell type (B cells). This is compatible with the results of the light microscope thymidine-^{3}H studies of Fischman and Hay (28) on regenerating newt limbs. Their results led these authors to conclude that the definitive osteoclast is derived from a precursor cell type with characteristics similar to those of the mononuclear leukocyte.

Cytogenesis of the Labeled Cells

The results of the present study would seem to leave little doubt that the A and B cells are derived from mesenchymal elements accompanying the invading capillaries. Thus, recognizable primordial forms of both cell types are characteristically seen to be localized in pericapillary sites.

These labeled primordial forms of the A and B cells are linked to their respective labeled endosteal representatives by a series of intermediates exhibiting a progressive increase in cytoplasmic organization. Accepting the basic hypothesis that, at 1-hr postinjection period, thymidine uptake is primarily restricted to "osteoprogenitor cells" undergoing DNA synthesis preparatory to mitosis (7), we may reasonably assume, therefore, that the A and B cells may undergo active proliferation and cytodifferentiation throughout a large part of their developmental histories. Thus, the possibility suggests itself that in vivo these two cell types might be independently self-propagating.

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