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An electron-microscopic study of the bone-remodeling sequence in the rat

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Summary. A detailed chronological electron-microscopic study of the bone remodeling sequence has been performed in the rat based on a previously described model (Tran Van et al. 1982) in which the remodeling activity is synchronized. This allowed the observation of the cellular and extracellular events during the bone remodeling process, including the activation of the sequential process and the reversal phase, intermediate between osteoclastic resorption and osteoblastic formation. Most important is the fact that throughout the whole process cells with the morphological characteristics of mononuclear phagocytes have been observed in proximity or in contact with the bone surface and/or the various bone cells. Coated pits (receptor-mediated endocytosis) are frequently observed in close apposition to bone spicules and gap junctions are frequent between the cells. These observations suggest that, besides being likely candidates as osteoclast precursors, mononuclear phagocytes may play an important role in bone remodeling.

Key words: Bone remodeling – Osteoclast – Osteoblast – Mononuclear-phagocytes – Osteoclast precursors

Most electron-microscopic studies of the cells involved in bone remodeling have been limited to either a single cell type (Scott and Pease 1956; Scott 1967; Hancox 1972; Pritchard 1972) or all the cells found along the bone surface without integrating the results into a sequence of events (Luk et al. 1974). These limitations were essentially related to the asynchronism of the various remodeling sites, not allowing the observer to know with certainty the stage of the remodeling process of the particular area under study. The renewal of bone tissue is actually accomplished through an orderly sequence of events occurring at individual sites along the bone surface. This remodeling sequence involves the succession of various cell types accomplishing various functions. After a phase of activation (A) osteoclast

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precursors fuse into a multinucleated osteoclast which resorbs (R) the old bone; after an intermediate reversal phase (Rev), osteoblasts appear at the same site and form (F) new bone (Frost 1964; Baron 1973, 1977).

The present report provides a detailed chronological electron-microscopic description of a synchronized remodeling sequence based on a previously described model (Tran Van et al. 1982).

Materials and methods

Twenty-seven male Wistar rats weighing 200 ± 20 g, used in this study, were provided with a standard rodent diet (Ralston Purina Co.) and tap water ad libitum. They were housed in individual cages in rooms kept at 75° F with a 12 h/12 h light cycle.

Bone remodeling was induced along the periosteal bone surface as described previously (Tran Van et al. 1982): Twenty-four animals were subjected to extraction of the right maxillary molars and sacrificed in groups of three animals every day for up to 7 days and at 10 days after induction of the remodeling process. Three animals served as controls. Sacrifice was performed by intracardiac perfusion with Karnovsky's fixative solution (2% paraformaldehyde, 3% glutaraldehyde) at pH 7.2. The right mandibles (opposite to the extracted row of teeth) were dissected out, the buccal side of the molar area was isolated, separated into two fragments and left overnight in the same fixative at 4° C. They were then washed in 0.1 M cacodylate buffer, pH 7.2, and post-fixed in 1% osmium tetroxide. The specimens were embedded without decalcification in Epon (Polybed, Polysciences, USA). The area under study (buccal-periosteal surface between the buccal roots of the first and second lower molars) being too large for electron microscopy, semithin sections, 1 μm thick, were prepared and stained with toluidine blue (1%). Ultrathin sections were then cut in a selected area. These sections were double stained with uranyl acetate and lead citrate and examined with a Philips 201 electron microscope operated at 60 KV.

Results

In control animals (Fig. 1), the calcified bone along the periosteal surface is entirely covered by osteoid tissue with a typical mineralization front at the interface between calcified and non-calcified matrix. All along this osteoid tissue a regular layer of cells shows the usual characteristics of active osteoblasts. Osteoclasts and/or mononuclear phagocytes are usually absent in this area in control animals.

Two or three days after induction (Fig. 2), osteoid tissue no longer separates the periosteal cells from the calcified bone. The osteoblasts are flattened and elongated; their peripheral cytoplasmic extensions are reduced in size and number so that the cells look smoother than in the controls. The endoplasmic reticulum and the Golgi complex area are less developed. In addition, during this activation period, the cells along the bone surface are heterogeneous compared to the uniform population of active osteoblasts found in the controls. Cells with the known morphological characteristics of mononuclear phagocytes suggestive of phagocytosis lie close to the bone surface and between osteoblasts (Fig. 2). These cells reach the calcified bone surface by sending pseudopods between the osteoblasts. In the area facing the bone surface their plasma membrane shows many coated pits, often circumscribing calcified bone spicules. Cellular processes are sometimes observed inside the bone canaliculi in contact with the osteocyte's processes (Fig. 2). Towards the end of the activation phase (days 3–4 after induction), the membrane of the mononuclear phagocytic cells facing the bone surface shows more numerous coated pits and a marked increase in ruffling. Bone matrix mobilization is now prominent. There is also an increase in the number of mitochondria, lysosomes, and vesicles in these

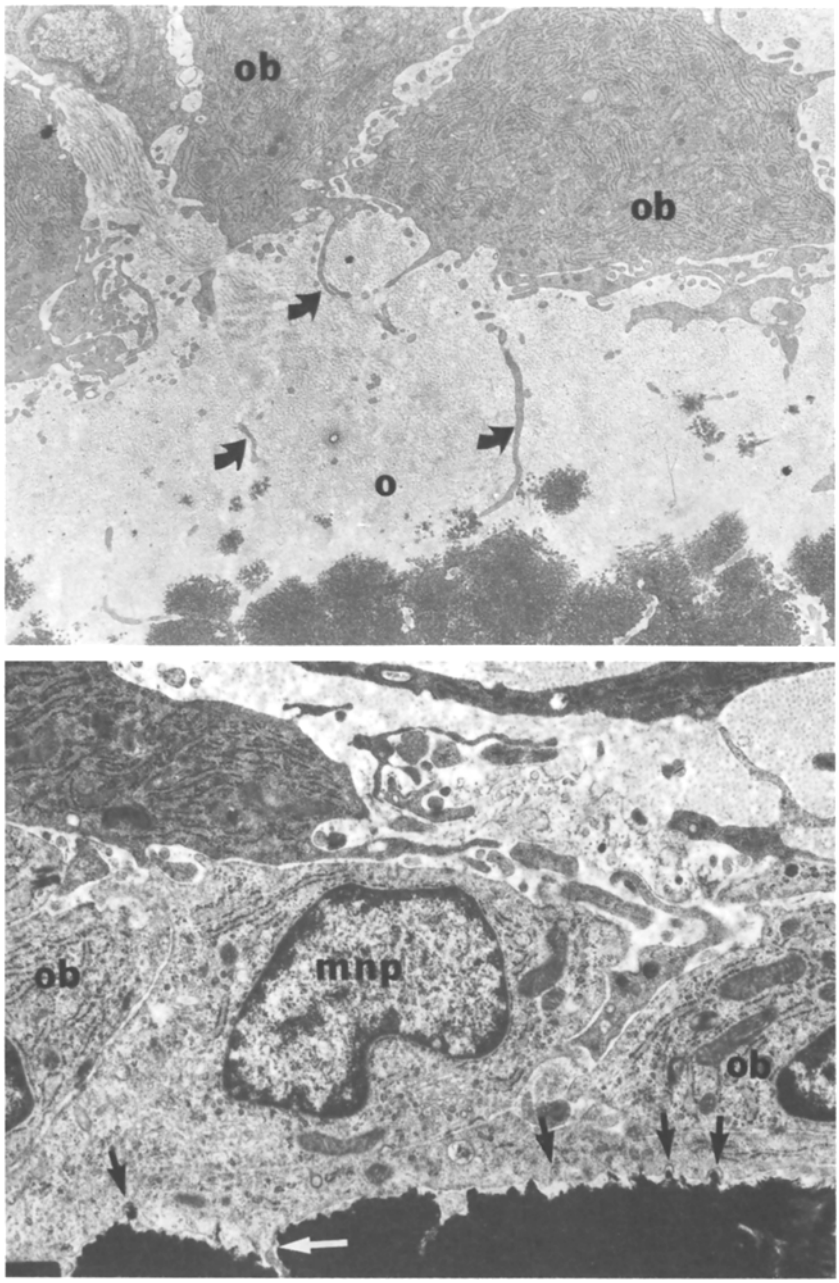

Fig. 1. Control animal. Calcified bone along periosteal surface entirely covered by osteoid tissue (*O*) lined with active osteoblasts (*ob*) showing extensive RER cisternae, large Golgi complex area, and numerous cytoplasmic extensions in the osteoid (*arrows*). Calcified bone as dark on lower part of micrograph. ×6,500

Fig. 2. Experimental animal during activation phase (2 days after induction). No osteoid tissue separating cells of calcified bone. Cells with the morphological characteristics of mononuclear phagocytes (*mnp*) reach bone surface by sending pseudopods between inactive lining osteoblasts (*ob*). Membrane of MNP-like cells in close apposition to bone surface; coated pits (*arrows*) in front of calcified bone spicules. *White arrow* points at contact between MNP-like cell and osteocytic process in canaliculus. ×10,000

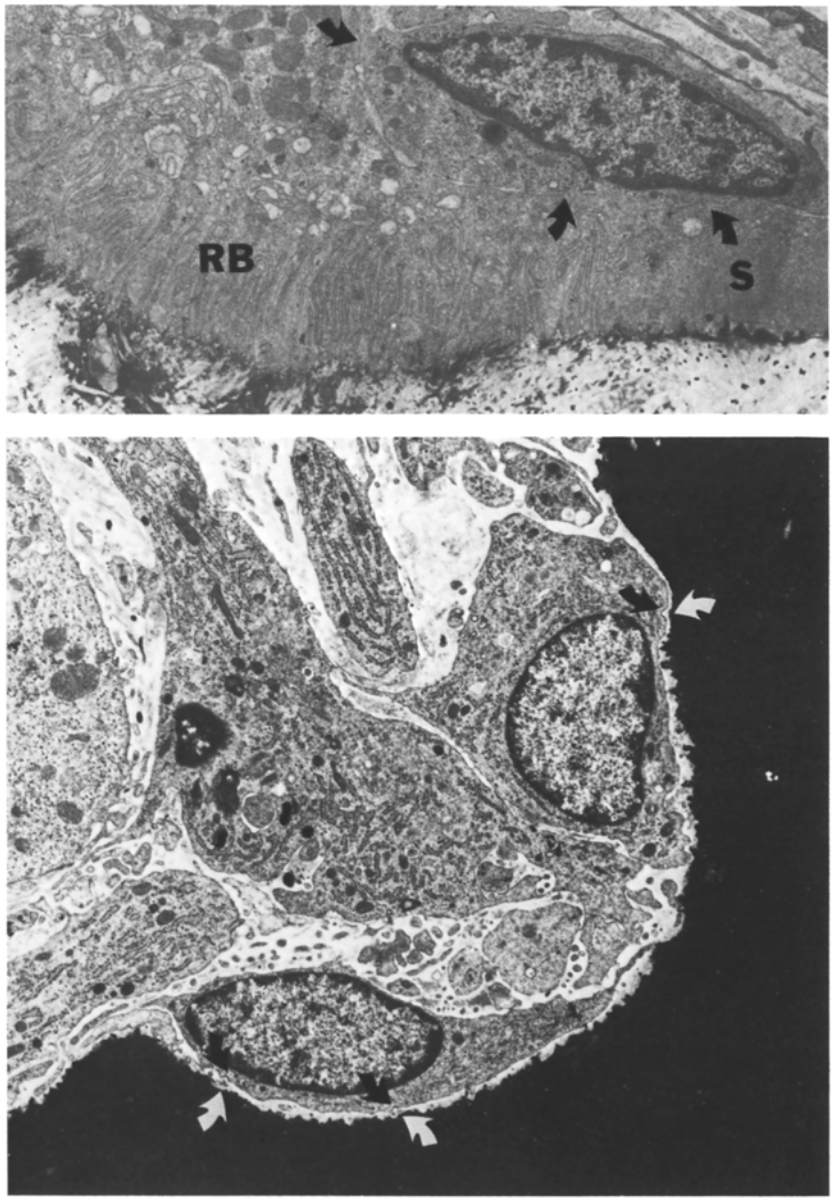

Fig. 3. Part of an active osteoclast with ruffled border (*RB*) and sealing zone (*S*); MNP-like cell, rich in electron-dense bodies and vesicles, in very close contact with osteoclast membrane (*arrows*). ×7,900

Fig. 4. Experimental animal, reversal phase (6 days after induction). Reversal lacunae with MNP-like cells in close contact with bone surface within Howship's lacuna. Numerous lysosomes, phagocytic vacuoles, and pseudopods. Numerous coated pits (*arrows*) along bone surface. ×5,200

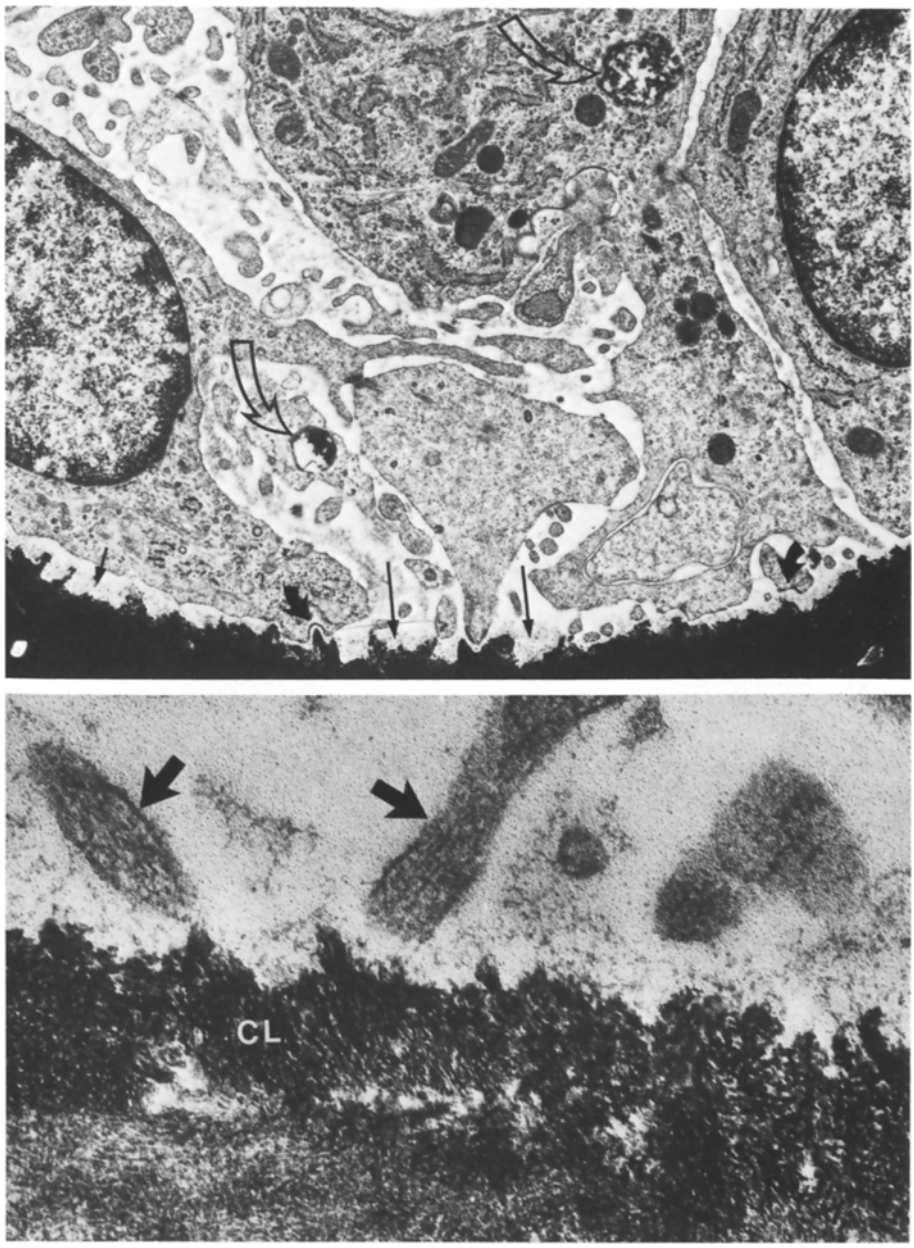

Fig. 5. Higher magnification of section close to that of Figure 4: MNP-like cells in contact with bone surface during reversal phase; numerous lysosomes and phagosomes, some containing crystalline material (*open arrows*); notice also coated pits facing bone spicules (*closed arrow*) and granular coat along bone surface (*thin arrows*). ×11,900

Fig. 6. Experimental animal, reversal-formation phase (7 days after induction): Calcification of cement line (*CL*) at bone surface and development of osteoblastic processes (*arrows*) containing microfilaments. ×74,000

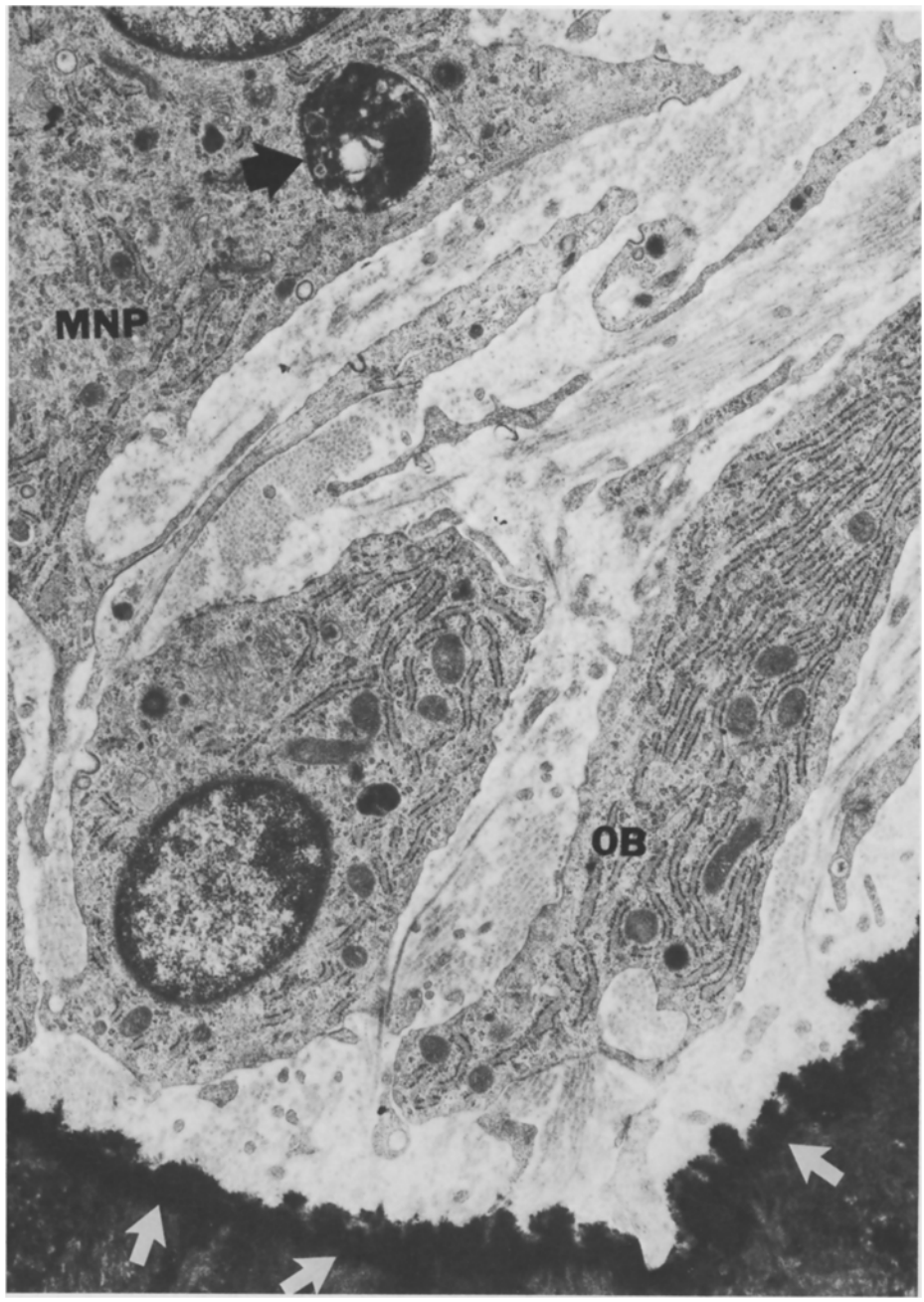

Fig. 7. Experimental animal, formation phase (10 days after induction). Osteoblasts (*OB*) appear within lacunae, osteoid deposited along calcified cement line (*arrows*); MNP-like cells (*MNP*) with large secondary lysosomes (*thick arrow*) at some distance from bone surface. ×12,500

cells. At that time, the cells look very similar to an osteoclast, except for a lack of multinucleation and of well differentiated perinuclear Golgi saccules. Large homocellular and heterocellular gap junctions as well as intracytoplasmic gap vesicles are frequent among osteoblasts, lining cells, and mononuclear phagocytes.

During the resorption phase (4–5 days after induction), most of the bone surface is covered by typical mature multinucleated osteoclasts. The bone surface underlying the ruffled border appears rough and shows numerous mobilized bone crystals. The bone surface facing the sealing zone is also irregular in contour but the plasma membrane is very closely apposed to it. At the periphery of the sealing zone the osteoclast membrane is detached from the bone surface forming a pseudopod with numerous short cytoplasmic extensions and coated pits circumscribing calcified bone spicules. Opposite to the bone surface, the osteoclast is surrounded by numerous uncharacteristic mononuclear cells. However, these cells often have numerous lysosomes, vesicles, and free ribosomes and are frequently observed in close contact with the osteoclast membrane (Fig. 3).

At days 6–7 after induction, most of the osteoclasts are detached from the bone surface, and show numerous short cytoplasmic extensions. Howship's lacunae created by the osteoclast during the active resorption period are now occupied by mononuclear cells (Reversal phase) (Fig. 4), some in close apposition to the bone surface. They have most of the morphological characteristics of mononuclear phagocytes, i.e., numerous electron dense bodies and phagosomes, some loaded with crystalline material, many free ribosomes, and numerous pseudopods (Fig. 5). These mononuclear phagocytic cells also show numerous coated pits along their plasma membrane which are typically associated with calcified bone spicules when facing the bone surface (Fig. 5). Away from the bone, but still within these lacunae, there is a mixture of cell types, some showing a more prominent rough endoplasmic reticulum (Fig. 4). Gap junctions are often observed between these various cells. The bone surface is covered by a dense granular collagen-free layer towards the end of the reversal phase (Fig. 5). Later (7–10 days after induction) this granular collagen-free material calcifies and becomes the cement line (Fig. 6). At the same time, cells with all the morphological characteristics of preosteoblasts and osteoblasts appear within these lacunae and thin cytoplasmic extensions containing numerous microfilaments are situated along the calcified cement line (Fig. 6). Newly synthesized osteoid tissue is later observed on top of the cement line during the active formation phase (10 days) and the cells lining the bone surface are typical active osteoblasts. Mononuclear phagocytic cells are still present, but behind this osteoblastic layer (Fig. 7).

Discussion

This study is the first electron-microscopic chronological description of the bone remodeling sequence. The most interesting findings are based on the new ability that we have developed (Tran Van et al. 1982) to follow a bone surface during the entire sequence of events through a complete remodeling cycle, including the activation and reversal phases. Most important is the fact that throughout the whole process cells with the morphological characteristics of mononuclear phagocytes have been observed in proximity or in contact with the bone surface

and/or other bone cells. When in contact with the bone surface they constantly showed numerous coated pits facing the circumscribing calcified bone spicules; when in contact with other cells, they showed either gap junctions (with other mononuclear phagocytes, osteoblasts and/or preosteoblasts) or extensive ruffling of their plasma membrane (with other mononuclear phagocytes and/or osteoclasts). These observations suggest that, besides being likely candidates as osteoclast precursors (Jee and Nolan 1963; Marks and Walker 1976), mononuclear phagocytes may play a very important role throughout the whole bone remodeling sequence in cell-cell and cell-bone interactions.

In this model, the first osteoclasts appear 3 days after induction (Tran Van et al. 1982). It is therefore possible to observe the cellular events occurring prior to resorption, i.e., during the activation phase. After cessation of matrix synthesis by osteoblasts and calcification of previously synthesized osteoid, cells with all the morphological characteristics of mononuclear phagocytes (Goldberg and Ravinovitch 1977) appear, reaching the bone surface by long pseudopods. Besides corresponding to the classical description of mononuclear phagocytes, these cells are also similar to the "osteoclast precursors" described by Scott (1967) and Luk et al. (1974). However, their appearance along the bone surface just before resorption makes it more likely that they actually are osteoclast precursors. In addition, the observation of coated pits along their plasma membrane, often associated with calcified bone spicules, suggests that these cells are specifically internalizing a substance located at the bone surface through receptor-mediated endocytosis (Goldstein et al. 1979). These observations could be the morphological counterpart for the previously reported chemotactism of monocytes for bone (Mundy et al. 1977; Kahn et al. 1978; Minkin et al. 1981) and would fit Chambers' (1980) hypothesis of a phagocytic recognition by macrophages of the bone to be resorbed. The fact that these mononuclear phagocytic cells then show increased ruffling of their membranes both along the bone surface, where morphological evidence of resorption becomes prominent, and at sites not facing the bone surface where short cellular processes are intricated with processes from adjoining cells compares with a very similar observation reported by Sone et al. (1981) during induced fusion of alveolar macrophages in culture. This observation therefore raises the possibility that this ruffling is part of the fusion process which leads to the numerous multinucleated osteoclasts observed 1 to 2 days later at the same site (Tran Van et al. 1982). The process of fusion is most likely to continue throughout the active resorption phase by osteoclasts and numerous mononuclear cells, some mononuclear phagocyte-like, are often in close contact with the osteoclast's membrane. A similar observation has been made by Schultz et al. (1977) in Paget's disease osteoclasts, Kurihara and Enlow (1980) who described them as "osteoclast companion cells", and Rifkin and Heigh (1980) who consider them as "fibroblast-like" cells involved in collagen digestion. Some of these cells are likely to be the osteoclast precursors in the process of fusing with an already differentiated osteoclast. They may also correspond to the cells showing a Fc receptor described by Jones et al. (1981) around osteoclasts and/or the cells rich in N-Acetyl β glucosaminidase described by Dorey and Bick (1977). Once the osteoclasts become detached from the bone surface at the end of the resorptive phase, they leave Howship's lacunae in which mononuclear phagocytic cells once again come in

contact with the bone, during the reversal phase. Our observations suggest an intense specific phagocytic process through receptor-mediated endocytosis during this phase. Here again, this could be the morphological counterpart for the observation that resorbing bone is chemotactic for monocytes (Mundy et al. 1978). In this case, however, and unlike what happens during the activation phase, this chemotactic activity could be related with the reversal phase more than with the flow of osteoclast precursors preceding or accompanying the resorption phase. The cells observed here could therefore be different from these latter and/or play a different role in the remodeling process. Although apparently fitting with Heersche's hypothesis (1979) that two different cell types are involved in the bone resorption process, our observations show that this macrophage activity does not involve digestion of collagen left by osteoclasts: it is not part of the resorptive process but instead of the reversal and coupling activities (Baron et al. 1980). The presence of mononuclear phagocytes during the reversal phase and of a granular coat along the bone surface just before the appearance of osteoblasts may suggest that these cells and/or this material play a part in the local coupling process.

In summary, this study describes the complete sequence of cellular and extracellular events taking place during the remodeling process. For the first time, it has been possible to observe the activation and reversal phases in electron microscopy, in addition to the classical description of resorption by osteoclasts and formation by osteoblasts. Our results suggest that mononuclear phagocytic cells play an important role during all the different steps of the bone remodeling process and not only as potential osteoclast precursors.

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