

## Induction of abundant osteoclast-like multinucleated giant cells in adjuvant arthritic rats with accompanying disordered high bone turnover

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**Summary.** The development of an *in vivo* system for investigating osteoclast differentiation is important because molecular events occurring *in vivo* can be observed during the differentiation of the authentic osteoclasts. In adjuvant arthritic rats, an experimental model of human rheumatoid arthritis, extensive bone resorption is observed in the distal diaphysis of the tibia. In the area of extensive bone resorption, it is always accompanied with clusters of numerous multinucleated giant cells (MGCs) as well as bone-resorbing osteoclasts. Here we characterized the morphological properties of these MGCs with the use of enzyme-histochemical and immunohistochemical techniques. Extensive destruction but also a marked formation of the inner and outer bone surfaces were the predominant features in the tibiae of such arthritic rats 4 weeks after the adjuvant injection. Numerous MGCs were frequently clustered in the bone marrow spaces located apart from the bone matrices. Although the MGCs lacked ruffled borders, these cells were rich in mitochondria and vacuoles. These multinucleated cells revealed a positive reaction for tartrate-resistant acid phosphatase but a negative reaction for non-specific esterase staining. Most of these MGCs expressed the Kat 1-antigen, an immunological marker specifically expressed on the cell surface of rat osteoclasts. In a dentin resorption experiment using a cluster of MGCs excised from the bone marrow tissues of the tibial distal diaphyses of rats with adjuvant arthritis, many resorption lacunae were formed on dentin slices after a 3-day culture. These results suggest that the majority of the MGCs are osteoclasts but not macrophage polykaryons.

**Key words:** Adjuvant arthritic rat, Osteoclast, Multinucleated giant cell

### Introduction

Osteoclasts are hematopoietic in origin (Udagawa et al., 1990; Suda et al., 1992) and *in vitro* cell culture systems for forming osteoclast-like multinucleated cells have been established in several animal species. *In vitro* studies have demonstrated the involvement of various cytokines in the regulation of osteoclastogenesis. In contrast to the ease of handling provided by the *in vitro* culture system for osteoclast differentiation, it is highly complex and difficult to examine osteoclastogenesis *in vivo*. A reproducible *in vivo* model of osteoclastogenesis has been developed using ovariectomized rodents (Jilka et al., 1992), which provides an appropriate system for studying post-menopausal osteoporosis. However, tracing osteoclast formation is extremely challenging even when an extensive morphological search is performed, because the increase in the number of osteoclasts occurs in the whole skeleton. This aspect makes it impossible to distinguish newly formed osteoclasts from pre-existing osteoclasts by the estrogen deficiency.

Adjuvant arthritic rats are known to develop polyarthritis induced after a single intradermal injection with complete Freund's adjuvant and thereafter demonstrate redness and swelling in the ankle joint, and the formation and destruction of bones in the hind paws occur then (Pearson and Wood, 1963; Akamatsu et al., 1966; Zahiri et al., 1969; Mori et al., 1970; Mohr et al., 1974). Since these clinical symptoms are similar to those seen in human rheumatoid arthritis (Pearson and Wood, 1959), adjuvant arthritic rats have been frequently used as an experimental model of rheumatoid arthritis (Ackerman et al., 1979; Taurog et al., 1985; Binderup, 1986; Del Pozo et al., 1990; Russell et al., 1993; Conway et al., 1995).

One of the features of adjuvant arthritis is the extensive destruction of the bones. It has been reported that such bone destruction is performed by the multinucleated giant cells (MGCs), osteoclasts, in the bone marrow cavity of the tibial diaphysis in adjuvant

arthritic rats (Tomoda et al., 1986). In the bone marrow cavity of the diaphysis, a cluster of MGCs are present, these MGCs clusters are located apart from the bone matrices. However the characterization of these cells has not been further established.

Here we examined the phenotype of the MGCs and the functional potential of these cells as osteoclasts, and we obtained evidence that MGCs meet several criteria of the osteoclasts with potential bone resorbing function.

## Materials and methods

### Induction of adjuvant arthritis

Adjuvant arthritis was induced by a single intradermal injection of 25 mg/kg of heat-killed *Mycobacterium butyricum* (Difco Lab., Detroit, MI, USA) suspended in mineral oil at the tail base of female Lewis rats (Seac Yoshitomi, Fukuoka, Japan) weighing 100-110 g, under ether anesthesia. At the same time, control rats were injected with mineral oil alone.

### Tissue preparations

Four weeks after the adjuvant injection, the animals were anesthetized with nembutal (Abbott Lab., North Chicago, IL, USA) and fixed by intracardiac perfusion with PLP fixative (0.01M sodium-meta-periodate/0.075M L-lysine-HCl/2% paraformaldehyde/0.0375M phosphate buffer, pH 6.2) or half-Karnovsky fixative (2% paraformaldehyde/2.5% glutaraldehyde/0.1M phosphate buffer, pH 7.4) for light or electron microscopy, respectively. Tissue blocks (ca. 2×4 mm) were taken from the hind paws (tarsal bones and tibia en bloc) and were then further immersed in the same fixative for 6 hours at 4 °C. After several rinses with phosphate buffer, the tissue blocks were demineralized in 10% EDTA for 3-4 weeks at 4 °C. These tissue blocks were dehydrated, and embedded in paraffin. Serial sections (5 µm in thickness) were prepared for light microscopy. For the electron microscopy, tissue blocks were embedded in 5% agar, followed by the preparation of 200 µm-thick sections using a Microslicer (Dosaka EM Co., Kyoto, Japan). The slices were postfixed with 1% osmium tetroxide for 1 hour and then stained with 2% uranyl acetate for 1 hour, dehydrated in a graded ethanol series, and embedded in Epon 812 (Taab Lab., Berkshire, UK). Ultra-thin sections were stained with lead citrate and examined with an electron microscope (JEOL 1210, JEOL, Tokyo, Japan).

### Histochemistry

Paraffin sections (5 µm in thickness) were stained for tartrate-resistant acid phosphatase (TRAP) or non-specific esterase (NSE) activity at 37 °C for 30 minutes in a moist chamber with the use of a leukocyte acid

phosphatase kit (Sigma Chemical Co., St. Louis, MO) or alpha naphthyl acetate esterase kit (Sigma). TRAP-positive cells, which appeared as dark red cells, were identified as being of the osteoclast lineage. NSE-positive cells, which appeared as black cells, were identified as being of the cell-lineage of monocytes/macrophages.

### Immunohistochemistry

The expression of Kat 1 antigen, which is specifically expressed on the osteoclasts of rats, was examined on paraffin sections (5 µm in thickness) using a monoclonal antibody (mAb) Kat 1 as previously reported, by the pre-embedding method (Kukita et al., 1994a). As a negative control, the monoclonal antibody En 3 was substituted for the mAb Kat 1, since En 3 is of the same immunoglobulin class as the mAb Kat 1 (IgM) but has no reactivity against bone tissues (Inai et al., 1991). The Kat 1 antibody was injected into the peritoneal cavity (100 mg/kg) of rats with adjuvant arthritis before sacrifice. The tissue blocks were fixed in PLP fixative and embedded in paraffin. After the preparation of 5 µm-thick sections, the sections were preincubated with 3% normal donkey serum in phosphate-buffered saline (PBS) for 30 minutes at room temperature (RT) for blocking the nonspecific binding. They were then incubated with biotinylated donkey anti-mouse IgM (Jackson Immunoresearch, West Grove, PA) diluted to 1:200 in PBS for 30 minutes at RT. Finally, the sections were incubated with horseradish peroxidase-conjugated avidin-biotin complex (Vectastain ABC kit, Vector Labs, Burlingame, CA) for 30 minutes at RT. Antibody-antigen complex was visualized by diaminobenzidine (DAB substrate kit, Vector). To inhibit endogenous peroxidase, the sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 30 minutes before incubation with the secondary antibody. The sections were counterstained with methylgreen. TRAP staining was performed on some tissue sections which had already been positively stained for mAb Kat 1.

### Cell culture and dentin resorption experiment

The bone resorption activity of MGCs was examined by a dentin resorption experiment as follows: 10 bone marrow tissue blocks (ca. 1 mm<sup>3</sup>) with abundant MGCs were extracted aseptically from the tibial distal diaphyses of adjuvant arthritic rats. The tissue areas containing numerous MGCs could be easily distinguished by their grayish color. Six tissue blocks were suspended separately in 200 µl of alpha minimum essential medium (αMEM) containing 15% fetal calf serum (FCS) in the wells of 24-well multiwell culture plates. Each cell suspension was placed on a human dentin slice or plastic culture wells. After 20 minutes of preculture, 300 µl of αMEM containing 15% FCS was added to each well. The MGCs were incubated for 72 hours at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>

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and 95% air. The MGCs with dentin slices were fixed in citrate-formaldehyde-acetone (3% formaldehyde/65% acetone), stained with TRAP and then examined with a light microscope. Next, the slices were ultrasonicated in 0.25M ammonium hydroxide to remove the cell components and for the examination of resorption lacunae, stained with Meyer's hematoxylin, and then examined for any pit formation by light microscopy. The remaining 4 tissue blocks extracted from the tibial distal diaphyses of the adjuvant arthritic rats were fixed with 4% paraformaldehyde in 0.1M phosphate buffer, dehydrated, embedded in paraffin, cut in 5  $\mu$ m serial sections, and stained with hematoxylin and eosin. The multinuclear cells adhering to the trabecular bone were identified as osteoclasts, while other multinuclear cells detached to trabecular bones were identified as MGCs. Three sections were randomly selected from the serial sections of each tissue block. By observing each section under the microscope at the magnification of  $\times 100$ , numbers of osteoclasts and MGCs having more than three nuclei were counted. Ratio of MGCs was calculated from the numbers of osteoclasts and MGCs in each section.

#### **Results**

Adjuvant arthritis was clinically examined 2 weeks after the adjuvant injection for the degree of redness and swelling of each hind paw. The control rats did not exhibit any clinical symptoms of adjuvant arthritis. The extensive destruction and marked formation of the inner and outer bone surfaces of the hind paws (Fig. 1a) were the predominant features in the arthritic rats 4 weeks after the adjuvant injection. The outer periosteum showed an irregular bone formation in the vicinity of the tibia-calcaneus joint (Fig. 1b). Numerous osteoclasts and MGCs were observed in the bone marrow cavity of the tibial epiphysis. Numerous osteoclasts were observed in the resorption lacunae at the trabecular bone surfaces (Fig. 1c). The MGCs were frequently clustered in the bone marrow spaces apart from the bone matrices (Fig. 1d). Although these MGCs had no ruffled borders, they had abundant mitochondria and vacuoles (Fig. 2). Such an abundance of mitochondria and vacuoles was similar to the ultrastructural observations of osteoclasts resorbing bone.

To examine the characteristics of the clustered MGCs in the bone marrow spaces, we performed two-enzyme histochemistry. The MGCs showed a positive reaction for TRAP (Fig. 3a) and a negative reaction for NSE (Fig. 3b). Numerous TRAP-positive mononuclear cells were observed around the MGCs which stained positively for TRAP (Fig. 3a), while a number of NSE-positive mononuclear cells was also seen around the MGCs which stained negatively for NSE (Fig. 3b). We performed double staining of the same tissue sections for TRAP and NSE. As shown Fig. 3c,d, the MGCs positive for TRAP were negative for NSE. The mononuclear cells expressing a high level of TRAP

were negative for NSE, while the mononuclear cells expressing a high level of NSE were negative for TRAP. Interestingly, the mononuclear cells expressing a lower level of TRAP were also stained for NSE at a lower level. These data show the presence of common progenitor cells for macrophages and osteoclasts in these tissues of high bone turnover in adjuvant arthritic rats.

We next used the mAb Kat 1 which recognizes the cell surface antigen specifically expressed on rat osteoclasts but does not react with macrophage polykaryons as well as macrophages. The mAb Kat 1 stained positively on both the basolateral membrane surface of the osteoclasts attached to the trabecular bone (data not shown) and on the cell surface membrane of the MGCs in the adjuvant arthritic rats (Fig. 4a). In the sequential staining of TRAP and mAb Kat 1 on the same section, the majority of the MGCs stained positively for both TRAP and mAb Kat 1 (Fig. 4b), and  $72\pm 3.4\%$  of the TRAP-positive MGCs stained positively for mAb Kat 1. In the negative control for the pre-embedding method, neither osteoclasts nor MGCs stained for the control monoclonal antibody (Fig. 4c). These data suggest that MGCs are closely related to authentic osteoclasts.

To investigate the function of the MGCs observed in the bone marrow spaces of the rats with adjuvant arthritis, clumps of these MGCs were carefully excised and suspended and then seeded on plastic culture wells or dentin slices. On the plastic culture wells, numerous osteoclast-lineage, osteoclast-like multinucleated cells and preosteoclast-like mononuclear cells were identified by cytochemical staining for TRAP (Fig. 5a) and immunocytochemical staining with the mAb Kat 1 (Fig. 5b). When the cells were cultured on dentin slices, a large number of resorption lacunae were formed on these slices, as shown in Fig. 6. The percentage of pit numbers/TRAP-positive multinucleated cells was about 75%. To estimate the number of MGCs and osteoclasts contained in the tissue blocks used in the dentin resorption experiment, we examined 4 tissue blocks by means of serial paraffin sections stained with hematoxylin and eosin. As a result,  $83\pm 4.2\%$  of the multinucleated cells were identified as MGCs since they were detached from the trabecular bone. The remainder were identified as osteoclasts since they were attached to the trabecular bone surfaces. These data strongly suggest that the MGCs observed in the clumps in the bone marrow space were closely related to osteoclasts bearing the potential of bone resorbing function.

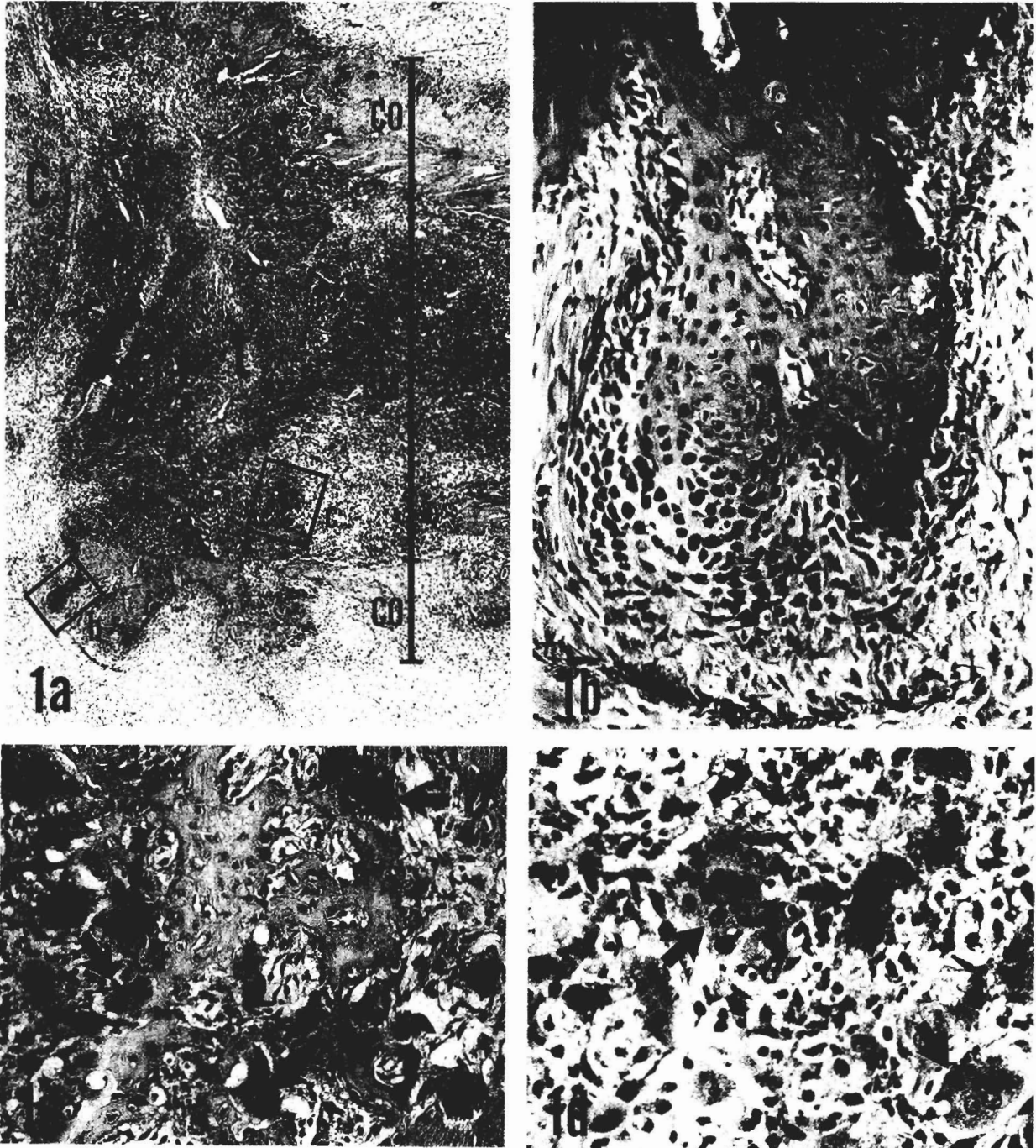
#### **Discussion**

Both extensive bone destruction and periosteal new bone formation of the hind paw were the predominant features seen in the adjuvant arthritic rats of the present study, as previously reported by many investigators (Pearson and Wood, 1963; Zahir et al., 1969; Mori et al., 1970; Mohr et al., 1974). Bonnet et al. (1993) reported that the decrease in the metaphyseal femur

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might be caused by a defect in bone formation but not of resorption, because the indices of bone formation showed marked decreases but those of bone resorption were not significantly changed as assessed by a bone

morphometric analysis. However, Binderup (1986) reported an enhancement of bone resorption in the adjuvant arthritic rats because of a decrease in the dry weight of tibial metaphyses and a concomitant increase



**Fig. 1.** a. Histological observation of distal diaphysis of the tibia in adjuvant arthritic rats. The area enclosed by the rectangles (b and c) are shown at a higher magnification in Figs. 1b and 1c, respectively. BM: bone marrow cavity; CO: compact bone; asterisk: joint cavity of calcaneus(c)-tibia(t) joint. b. An irregular bone formation of the outer periosteum in the vicinity of the tibia-calcaneus joint. c. A large number of osteoclasts (arrows) are observed in the lacunae of the trabecular bone surfaces. d. Multinucleated giant cells (MGCs) (arrows) of various sizes and shapes are clustered in the bone marrow spaces. a, x 25; b, c, x 180; d, x 350

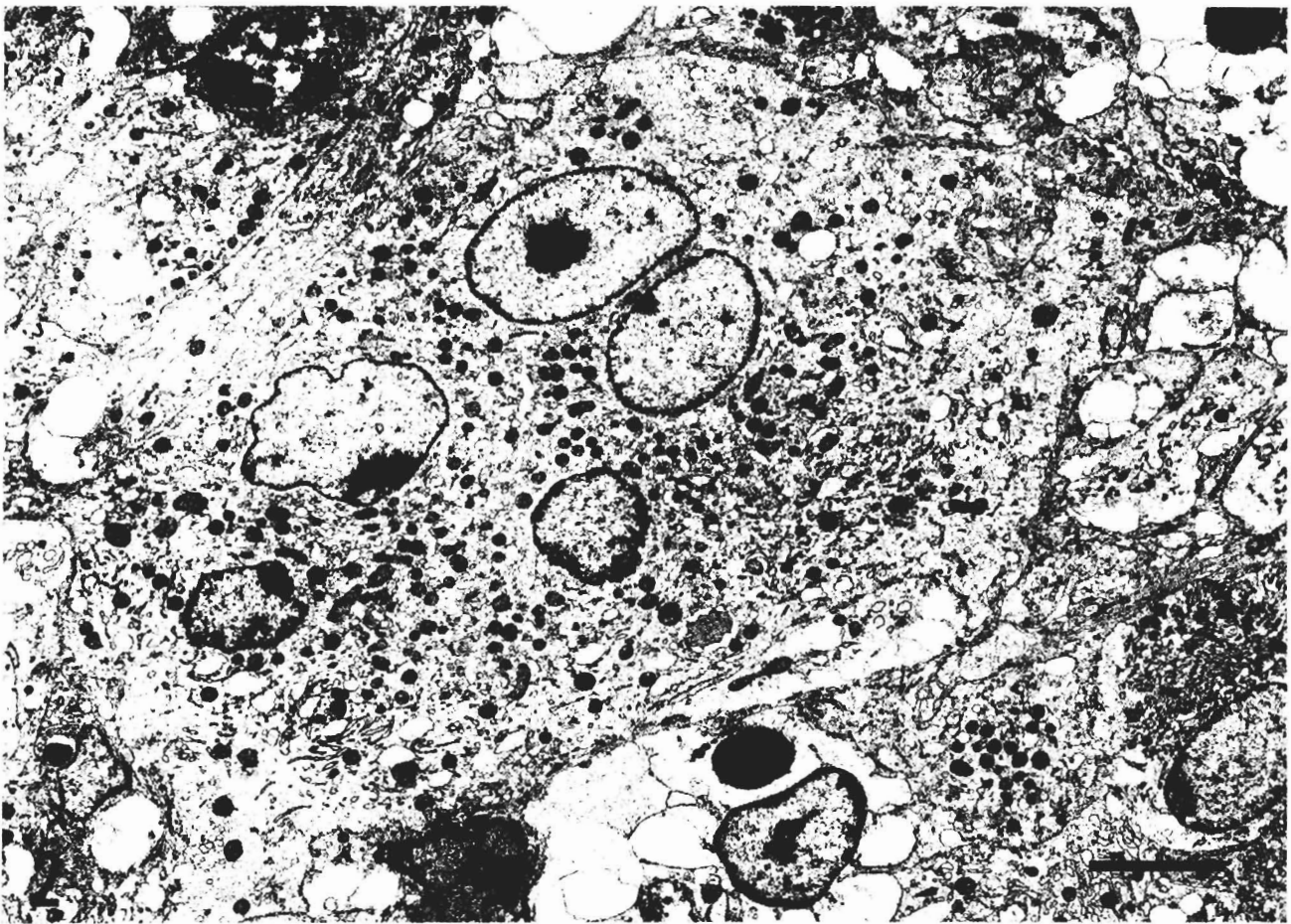
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in the urinary excretions of hydroxyproline and calcium. Since a large number of osteoclasts and MGCs were observed in the bone marrow spaces of the tibiae in the present adjuvant arthritic rats at 4 weeks after injection, we suggest that the extensive bone destruction may be due to an increase in osteoclastic bone resorption.

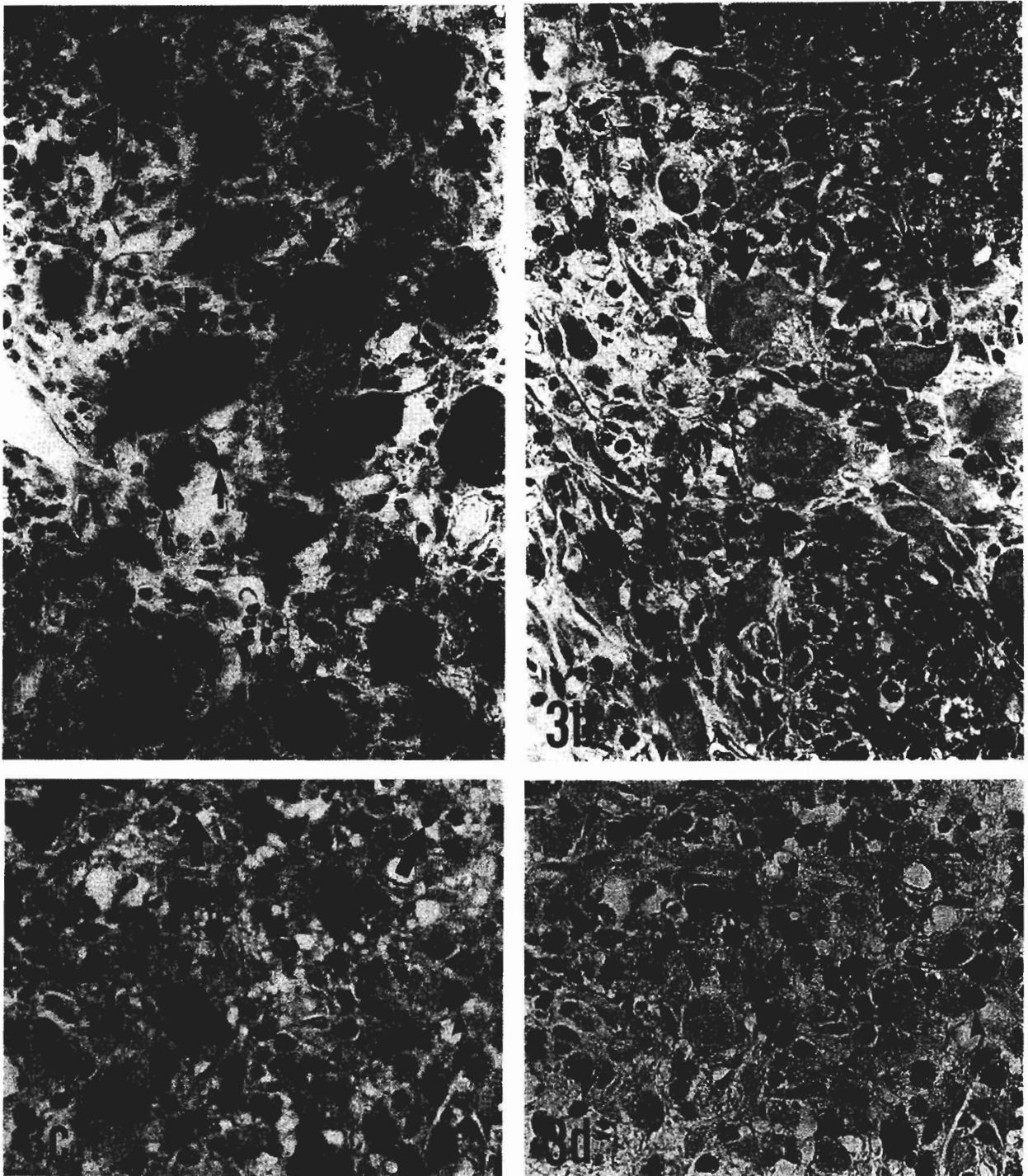
We examined the characteristics of MGCs to determine whether they were osteoclasts or macrophage polykaryons. The MGCs were frequently clustered in the bone marrow spaces apart from the bone matrices. The ultrastructural observation of the MGCs demonstrated that the cytoplasm of these cells was rich in mitochondria and vacuoles, and the multi-nucleus resembled those of osteoclasts, although the MGCs did not possess ruffled borders. These ultrastructural features are similar to those of the quiescent osteoclasts which Fukushima et al. (1991) reported.

Our enzyme-histochemical analysis showed that the MGCs were positive for TRAP but negative for NSE. The TRAP reactivity of MGCs was as intense as that of the osteoclasts which adhered to the trabecular bones. Marks and Chambers (1991) suggested that the MGCs

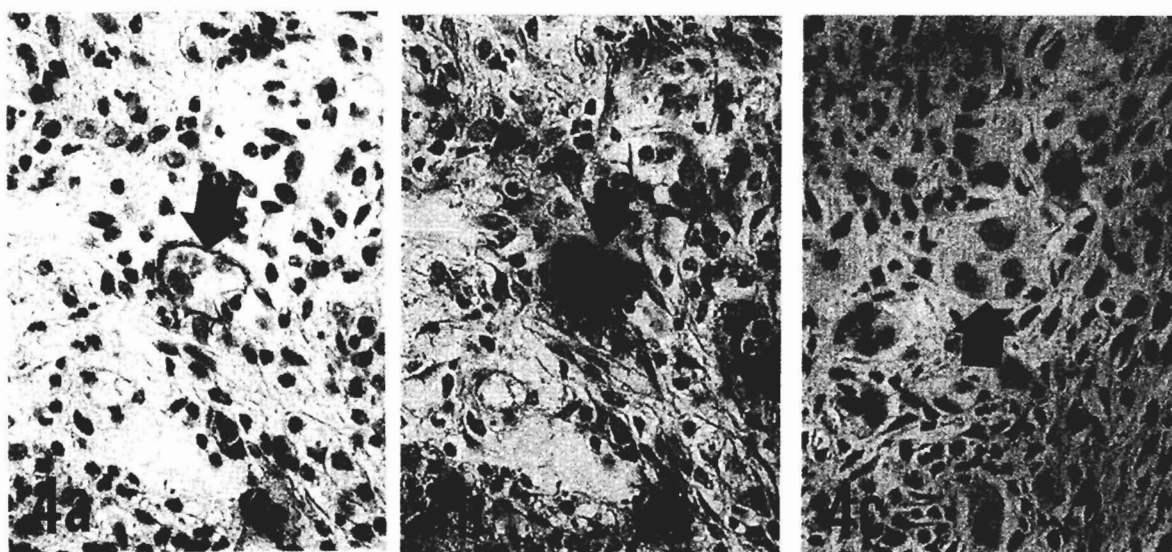
around the subcutaneously implanted bone particles were not osteoclasts, because the MGCs were stained only weakly for TRAP, whereas the host osteoclasts were strongly stained for TRAP. A high expression of TRAP is one of the important markers, although not a sufficient one, for identifying osteoclasts (Hattersley and Chambers, 1989; Modderman et al., 1991). We further examined the reactivity of the MGCs for NSE, a marker enzyme of monocytes/macrophages. It has been reported that authentic osteoclasts do not express NSE (Takahashi et al., 1994). In the present study, the MGCs revealed negative reactivity for NSE. Furthermore, many TRAP-positive and NSE-positive mononuclear cells were observed in the bone marrow spaces among these MGCs. Since it has been demonstrated that even mature macrophages can differentiate into osteoclasts under suitable conditions (Udagawa et al., 1990), the many TRAP-positive and NSE-positive mononuclear cells seen in our *in vivo* experimental system may thus be recruited as osteoclast progenitors in stress conditions such as adjuvant arthritis. The double staining of these bone tissues for TRAP and NSE



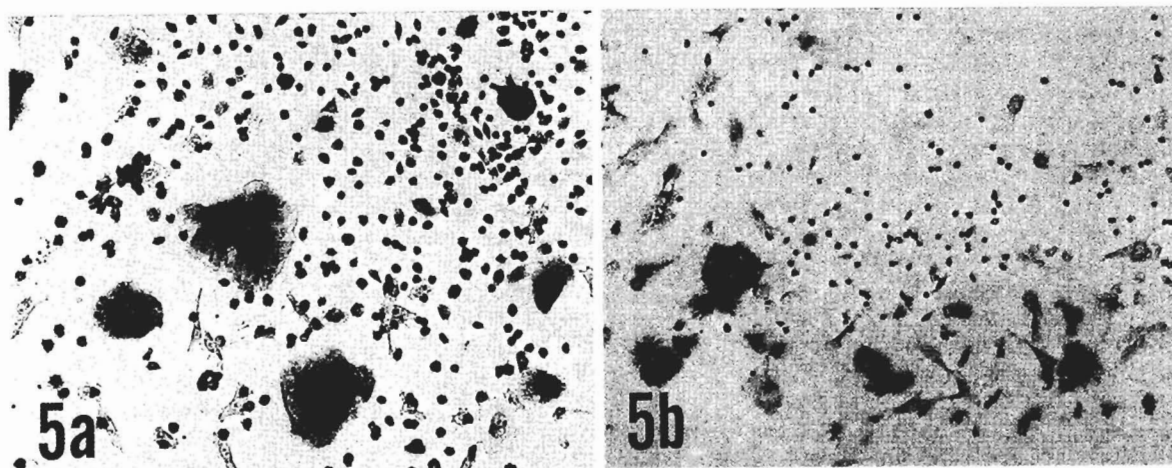
**Fig. 2.** Electronmicroscopic observation of MGC. The MGC contains abundant mitochondria and vacuoles but has no ruffled borders.  $\times 3,500$ . Bar:  $5 \mu\text{m}$ .

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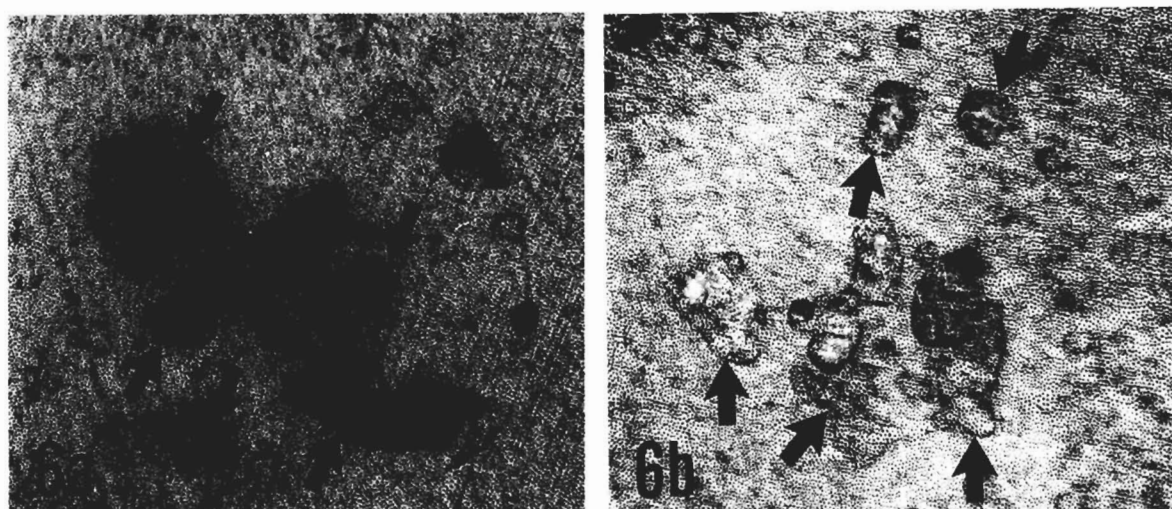
**Fig. 3.** An enzyme-histochemical staining of the TRAP and NSE activity in the bone tissue of adjuvant arthritic rats. **a.** MGCs (large arrows) and mononuclear cells (small arrows) are stained positively for TRAP. **b.** Mononuclear cells (small arrows) are strongly stained for NSE while MGCs (large arrows) are not stained for NSE. **c and d.** Double staining of the same tissue sections for TRAP and NSE. Mononuclear cells that are strongly stained for TRAP are not stained for NSE (large arrows) and mononuclear cells that are strongly stained for NSE are not stained for TRAP (small arrows). Mononuclear cells stained weakly for both TRAP and NSE are also observed (small arrowheads). MGCs are stained positively for TRAP but not for NSE (large arrowhead). a-d, x 350



**Fig. 4.** Sequential immunostaining of Kat1 and TRAP staining. **a.** The cell surface membranes of the MGCs are positively stained for the mAb Kat 1 (arrow). **b.** Kat 1-positive cells (Fig. 4a, arrow) are sequentially stained positively for TRAP (Fig. 4b, arrow). **c.** Immunostaining of the control antibody in the bone tissue in adjuvant arthritic rats. The MGCs are stained negatively for the control antibody (arrow). a-c, x 350



**Fig. 5a.** Cytochemical staining for TRAP. A large number of TRAP-positive mononuclear cells and multinucleated cells are observed. **b.** Immunocytochemical staining with the mAb Kat 1. Kat 1-positive mononuclear cells and multinucleated cells are observed. a, b, x 100



**Fig. 6.** Light microscopic observation of MGCs and pit formation on the dentin slices. **a.** MGCs (arrows) cultured on the dentin slice are stained positively for TRAP. x 100. **b.** A number of resorption lacunae (arrows) are observed on the dentin slices. x 100

revealed the presence of double-positive mononuclear cells at a significant level. These mononuclear cells expressing a low level of TRAP as well as a low level of NSE are considered to be the common progenitor cells of macrophages and osteoclasts. Mononuclear cells expressing a high level of TRAP but not NSE are likely to be preosteoclasts, while mononuclear cells expressing a high level of NSE but not TRAP are thought to be macrophages. In the bone marrow space with numerous MGCs, the differentiative conversion of macrophages into the mononuclear precursors of the osteoclasts, i.e., preosteoclasts, could occur in response to a stress condition (Nijweide and de Grooth, 1992) such as adjuvant arthritis.

We previously developed the mAb Kat 1 which recognizes rat osteoclasts in a highly specific manner. The mAb Kat 1 recognizes the cell surface antigen expressed on the cell surface of osteoclasts but not that of macrophage polykaryons or other hematopoietic cells involving macrophages (Kukita et al., 1994a,b; Xu et al., 1995; Kukita and Kukita, 1996). The cell surface membranes of MGCs stained positively for the mAb Kat 1 as did the basolateral surfaces of osteoclasts which adhered to the trabecular bone surfaces. Based on the findings of sequential staining using first the mAb Kat 1 and then TRAP on the same tissue sections, it became clear that about 70% of the TRAP-positive MGCs were also positive for the mAb Kat 1. In contrast, 30% of the MGCs stained negatively for the mAb Kat 1. These Kat 1-negative MGCs might therefore represent some different functional or differentiative state of the osteoclasts.

In the dentin resorption experiment, a large number of resorption lacunae were observed on the dentin slices. It has been reported that the MGCs in giant cell tumors formed many resorption lacunae on devitalized bone slices (Chambers et al., 1985), and that they acquired clear zones and ruffled borders after a culture on devitalized bone slices (Kanehisa et al., 1991), whereas they lacked clear zones and ruffled borders in vivo (Aparisi et al., 1977). Our data therefore suggest that MGCs of adjuvant arthritis could also function as bone resorbing osteoclasts.

In regard to osteoclastogenesis, it has been proposed that regulation by plural cytokines or by stromal cells is involved in osteoclastogenesis. For example, it has been demonstrated that interleukin (IL)-1, IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which are inflammatory cytokines, are important factors which promote the differentiation and activation of osteoclasts (Mundy, 1992). Further, in stromal cells, it has been reported that MC3T3-G2/PA6 and ST-2, which are bone marrow-derived stromal cell lines, and KS-4, which is an osteoblastic cell line, support the formation of TRAP-positive multinucleated cells in vitro. In contrast, MC3T3-E1, which is an osteoblastic cell line, does not support such a formation (Yamashita et al., 1990). It therefore seems that the formation of numerous MGCs and osteoclasts in adjuvant arthritis is also regulated by

various cytokines or stromal cells or both. In regard to stromal cells, the proliferation and differentiation of stromal cells which strongly promote osteoclastogenesis from undifferentiated mesenchymal cells might occur in the bone marrow.

The results obtained in the present study strongly suggest that the MGCs observed in adjuvant arthritic rats are indistinguishable from authentic osteoclasts and that they are not macrophage polykaryons. Consequently, such adjuvant arthritic rats are considered to be a very useful experimental model for studies of the function and differentiation of the osteoclasts, as well as a good experimental model of human rheumatoid arthritis.

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