Virchows Archiv (1994) 424:99–104

ORIGINAL ARTICLE

Yoshio Mii · Yoshizumi Miyauchi · Kanya Honoki
Toru Morishita · Shuichi Miura · Makoto Aoki
Susumu Tamai · Shigeru Tsunoda · Masaya Nishitani
Toshisuke Sakaki

Electron microscopic evidence of a viral nature for osteoclast inclusions in Paget's disease of bone

Received: 8 September 1993 / Accepted: 9 November 1993

Abstract Circumstantial evidence from electron microscopic and immunological studies support the view that Paget's disease of bone represents a slow virus infection. However, there is only limited information available regarding its electron microscopic, enzyme and immunocytochemical characteristics. Two cases were studied using electron microscopy with particular emphasis on the inclusions in osteoclasts. Detailed ultrastructural and cytochemical studies including immuno-electron microscopy were performed. Some osteoclasts demonstrated specific virus-like structures composed of aggregations of microtubules in the nucleus and cytoplasm. The structures were easily digested by trypsin or protease, and were sensitive to RNase, which provided substantial evidence of a proteinaceous nature and inclusion of ribonucleic acid. Immunocytochemical examination identified binding of anti-respiratory syncytial virus and anti-measles virus antibodies in the tissue obtained from one of the two cases examined. The presence of viral antigens in structures in the cytoplasm of Pagetic osteoclasts supports the theory of paramyxovirus involvement in this disease.

Key words Paget's disease of bone
Immunocytochemistry · Osteoclasts
Respiratory syncytial virus · Measles virus

Introduction

Paget's disease of bone is a common disorder believed to affect 1–3% of the population over 40 years old in Europe, Australia, New Zealand and the United States, whereas it is regarded as uncommon in Asia and Africa (Sissons 1966; Merkow and Lane 1990; Greenspan 1991; Siris et al. 1991). In Japan it rarely occurs and only about 150 cases have appeared in the literature (Hino et al. 1986; Takashima et al. 1988). Sir James Paget first described this localized bone disease of unknown cause in 1877 (Paget 1877). Subsequently, many studies on clinical and morphological characteristics have clarified its aetiology (Rebel et al. 1974; Milgram 1977; Mills et al. 1980; Siris and Canfield 1982; Mirra 1987). Since the discovery of specific inclusions in Pagetic osteoclasts in 1974 by Rebel et al. many researchers have provided electron microscopic evidence suggesting the possibility that they represent a virus or viruses, based on their ultrastructural appearance (Gherardi et al. 1980; Rebel et al. 1980; Singer 1980; Mills et al. 1981; Harvey et al. 1982). Recent immunofluorescence studies using antivirus antibodies (Baslé et al. 1986; Mills and Singer 1987) and more recent in situ hybridization techniques (Baslé et al. 1986; Baslé and Rebel 1987; Mills and Singer 1987) have lead to results strongly supporting a relationship between this disease's development and paramyxovirus infection. However, it has not been clarified whether the virus-like structures actually demonstrate an immune reaction against anti-viral antibodies because all previous immunocytological studies were performed at the light microscopic level. Thus, while many investigations have revealed morphological features and some circumstantial immunocytochemical evidence of viral involvement in this disease, but the immuno-electron microscopic characteristics of the inclusions have not been clarified. Recently, we observed virus-like inclusions in osteoclasts of bone tissue obtained from two patients with Paget's disease of bone. Detailed ultrastructural and cytochemical investigations were therefore performed to clarify the morphology and nature of the structures.

Y. Mii (✉) · Y. Miyauchi · K. Honoki · T. Morishita · S. Miura
M. Aoki · S. Tamai
Department of Orthopaedic Surgery, Nara Medical University,
840 Shijo-cho, Kashihara, Nara 634, Japan

S. Tsunoda · M. Nishitani · T. Sakaki
Department of Neurosurgery, Nara Medical University,
840 Shijo-cho, Kashihara, Nara 634, Japan

Materials and methods

A 54-year-old Japanese male and a 55-year-old Japanese female first presented themselves at our hospital in 1991 with dull pain in their low back regions and elevated serum alkaline phosphatase levels. Radiographic examinations of the skull, the lumbar vertebral bodies, sacrum and pelvis in case 1, and the sacrum and pelvis in case 2 revealed combinations of increased density and osteolytic areas, as well as areas with a honeycombed appearance. Scintigraphic examination was helpful in both cases to detect silent lesions which could not be found by routine radiographic examination. They were therefore diagnosed as suffering from Paget's disease and selected for a detailed electron microscope study utilizing enzyme and immunocytochemical approaches.

The specimens examined in the present study were obtained from the sacral bone of Case 1 patient and from the iliac bone of Case 2 at the time of biopsy. The specimens were cut into small blocks, fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4° C for 2 h and then washed with the same buffer containing 0.25 M sucrose at 4° C for 16 h. After decalcification in 2.5% EDTA solution with continuous stirring at 4° C for 48 h, they were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at 4° C for 1 h, followed by dehydration through a graded series of ethanols and embedding in Quetol 812 (Nisshin EM, Tokyo, Japan). The specimens were cut with an LKB-8800 ultratome and contrasted with uranyl acetate and lead citrate. They were observed under a JEOL 1200-EX electron microscope.

For enzyme digestions, enzymes were applied to thin sections from acrylate embedded specimens according to the technique described by Leduc et al. (1963). The enzymes used in this study were protease (Sigma, Type IV), 0.5% in distilled water, pH 7.4; trypsin (Sigma, Type I), 0.5% in 0.1 M TRIS-HCl buffer, pH 8.0; ribonuclease (RNase, Sigma Type I-A), 0.1% in distilled water, pH 6.8; deoxyribonuclease (Sigma Type IV), 0.5% in distilled water, pH 6.8. Thin sections on gold grids were incubated in medium containing each enzyme at 37° C from 15 min to 3 h. Control sections were treated with enzyme-free medium in the same manner.

For immunocytochemistry, the protein A-colloidal gold method was used according to the method of Timms (1986). Briefly, specimens were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1% cacodylate buffer (pH 7.4) under microwave irradiation for 30 s followed by an additional fixation procedure at 4° C for 1 h. The decalcifying procedure was carried out using the same method described above. Then the specimens, without post-fixation, were embedded in hydrophilic acrylate resin, LR-White (TAAB Laboratories Equipment, London, England) and polymerized. Ultra-thin sections mounted on nickel grids were floated on a drop of 0.1% bovine serum albumin (BSA)-containing phosphate buffered saline (PBS) for 15 min, and transferred to a drop of PBS containing anti-virus antibodies diluted to appropriate concentrations, for 2 h. After washing in PBS they were then incubated in protein A-colloidal gold complex (15 nm in diameter, E-Y Laboratories, San Mateo, Calif., USA) diluted in PBS for 1 h. Contrasting was with uranyl acetate before electron microscopic examination. For the immuno-cytochemical study, anti-parainfluenza virus total para-antibody, anti-respiratory syncytial virus (RSV) monoclonal antibody and anti-measles monoclonal antibody were used. All were purchased from Chemicon International, Inc. (Temecula, Calif., USA). For controls, anti-virus antibody free media were used for all the reactions.

Results

The specimens obtained from bone of the two patients show typical histological features of Paget's disease (Milgram 1977; Mirra 1987). Mosaic patterns in the bone trabecula and multinucleated giant cells with an osteoclastic appearance, localized at the bone trabecular surface, are observed. Small bone cavities near each osteoclast represent Howship's lacunae, where bone resorption occurs (Fig. 1). In other areas, the trabecula are surrounded by layers of osteoblasts. Both bone resorption and bone formation are coincidental findings and bone marrow cells and fat tissue are replaced by vascular fibrous tissue. No inflammatory cell infiltration is

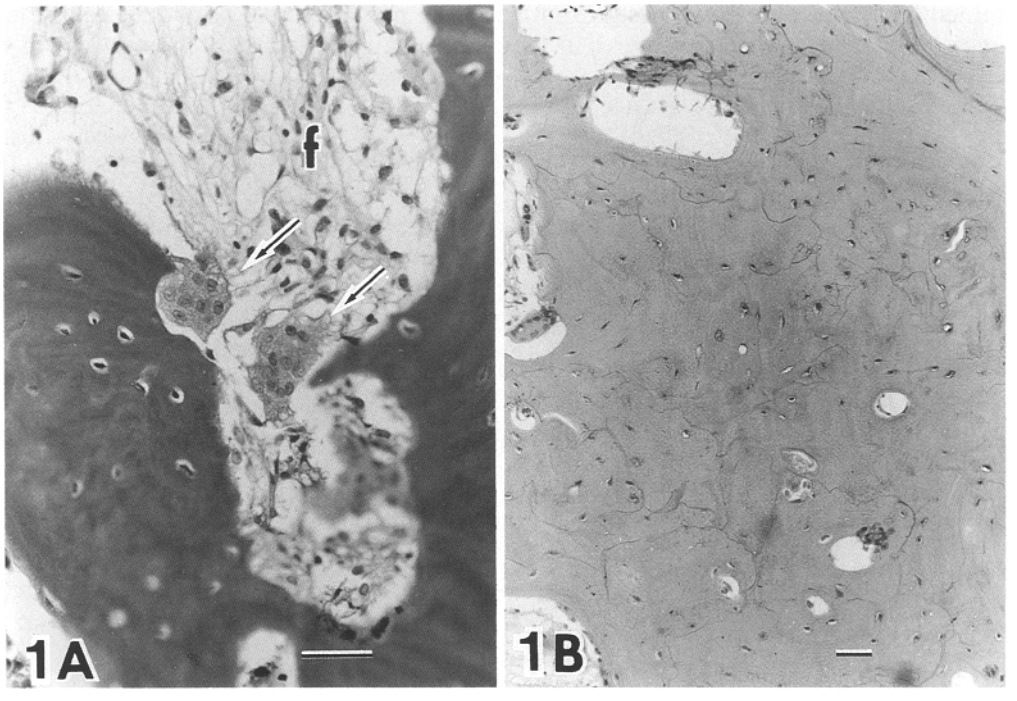

Fig. 1A, B Photomicrographs of haematoxylin and eosin stained section. Note active osteoclastic bone resorption (*arrows*) and marrow fibrosis (*f*) (**A**), and the classic mosaic pattern in the bone trabecula (**B**). Bar = 50 μm. × 400 (**A**) and × 200 (**B**)

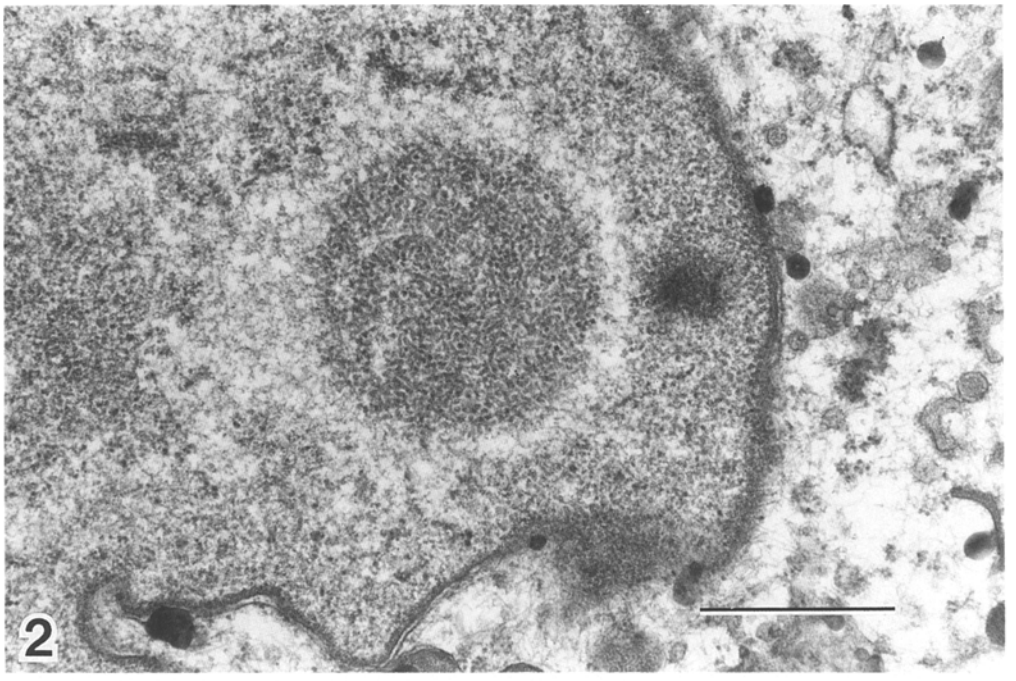

Fig. 2 Electron micrograph illustrating intranuclear inclusions in an osteoclast, showing packed aggregation of fine granular and microcylindrical material and apparent separation of areas by an electron-transparent zone. Bar = 1 μm. × 20 000

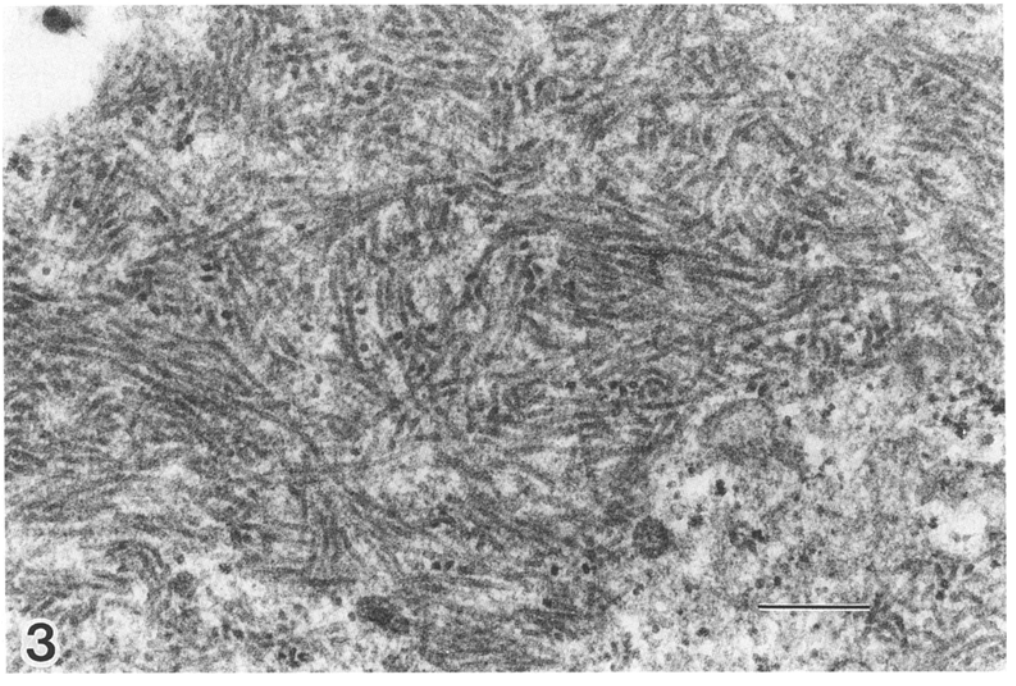

Fig. 3 Electron micrograph of an intracytoplasmic inclusion in osteoclast, showing an aggregation of microcylindrical structures. Bar = 0.5 μm. × 25 000

observed except for mononuclear cells, including histiocytic cells.

On electron microscopy round-shaped structures resembling nuclear bodies exist in the nuclei of some osteoclasts. At high magnification, these structures appear as packed aggregations of numerous microtubules and/or fine granules of about 16–20 nm in diameter separated by an electron-transparent zone from the normal nucleoplasm (Fig. 2). Abundant microtubules are generally distributed in a dispersed fashion and are only seldom grouped together in packed aggregations. The microtubules type of inclusions is also observed, essentially in the same form and size, in the cytoplasm of some osteoclasts (Fig. 3). In some cells, these inclusions are localized in small pockets within areas of cytoplasm while they occupy almost the whole cytoplasm in other cells. Occasionally the integrity of the nuclear membrane appears lost and the structures along with nuclear chromatin flow out into the cytoplasm (Fig. 4). The present results demonstrate the structures in the nucleus and those in the cytoplasm to have the same morphological character. No cells other than osteoclasts show any type of such inclusions.

Exposure to trypsin and protease was associated

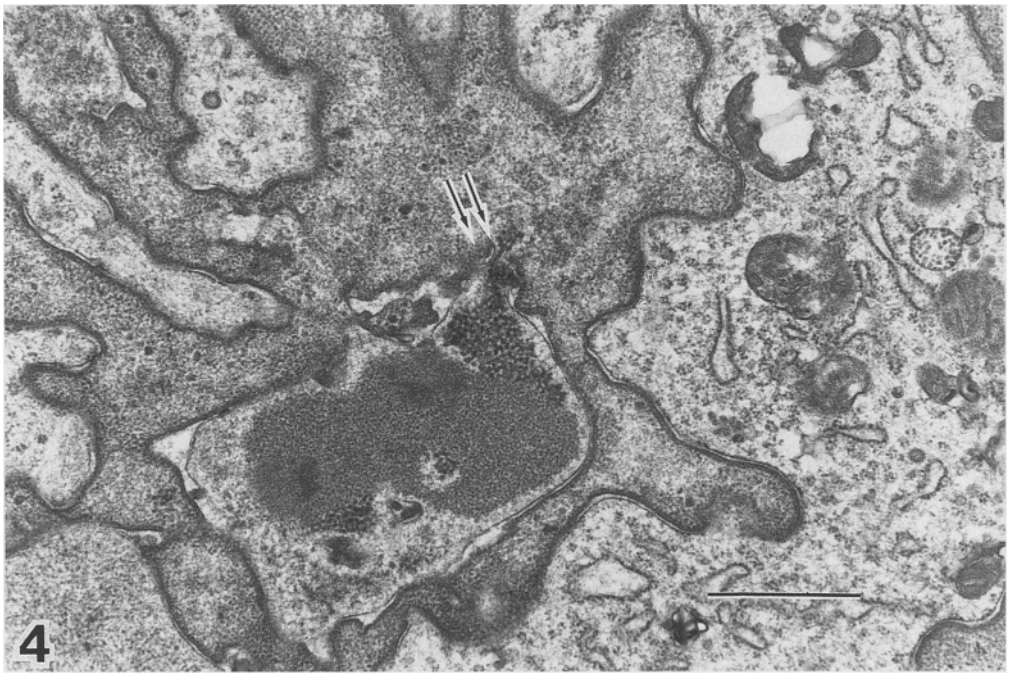

Fig. 4 Electron micrograph of intranuclear inclusions, showing apparent rupture of the nuclear membrane (*arrows*) with granular material both within the nucleus and in the adjacent cytoplasm. Bar = 1 μm. × 15 000

with marked change in the appearance of the structures. After treatment with these enzymes for 30 min, they were partially removed and had almost disappeared after a 1 h treatment. The structures were decreased in contrast intensity after exposure to RNase for 15 min and were recognized only as dispersed microtubules after 1 h. In contrast, they were not affected by DNase treatment, even after 3 h.

Immunocytochemistry reveals structures existing in the cytoplasm of osteoclasts in the bone tissue from case 1 to strongly bind anti-RSV and anti-measles virus-colloidal gold conjugates (Fig. 5). However, those in the nucleus were not labelled and the particles conjugated with anti-virus antibodies did not bind to any site in the cells of the specimens from case 2. Using antibody free control media, no positive labelling was observed with any specimen for immunocytochemical procedures.

Discussion

In our study we found characteristic virus-like structures in multinucleated giant cells with features of osteoclasts, such as a ruffled border and a clear zone. Virus-like structures were observed not only in the nucleus but also in the cytoplasm of the osteoclasts, their shapes, furthermore, being basically identical in the two cases. So what are these structures? Judging from their morphology, experienced researchers have suggested they might be nucleocapsids of paramyxovirus (Howatson and Fornasier 1982). Among the studies made to demonstrate that these structures are indeed viruses, the most reliable method involved immunological inspection using fluorescent antibodies and antibodies against measles, RSV, and sometimes the parainfluenza virus have demonstrated positive reactions in osteoclasts. However, the present electron microscope study is the first to demonstrate that the structures are viruses.

The fact that the incidence of this disease differs from country to country and from ethnic group to ethnic group suggests that it is an infectious disease. Reports that it sometimes shows familial development also supports the infection theory (Sofaer et al. 1983; Siris et al. 1991).

The results of the enzyme-digestion study clearly indicated a proteinaceous nature for the structures, as they were completely digested by trypsin or protease. Since it has been reported that paramyxovirus protein is sensitive to RNase on acrylate embedded sections, our results are in agreement in indicating the presence of ribonucleic acid in the structures (Gyorkey et al. 1972; Mii et al. 1988). However, we cannot solve the problem of why the structures showed a positive immunocytochemical reaction to two antibodies, and why the reaction was positive in only one case. Decalcification is necessary and prior to this procedure we used a microwave method which is considered to be effective at maintaining antigenicity in the specimens during fixation and decalcification (Mizuhira 1990). However, the possibility that the preparation procedures had a negative influence on antigenicity can not be discounted.

According to Baslé et al. (1987), the genetic mutation rate of RNA viruses is known to be high and the detection of antigens from different types of paramyxoviruses in Pagetic osteoclasts could well imply a polyviral infection. Since RSV and measles group viruses are closely related members of the RNA paramyxovirus family, the immunological finding of two closely related antigenic determinants (RSV and measles) together could mean that Paget's disease is either caused by the synergistic

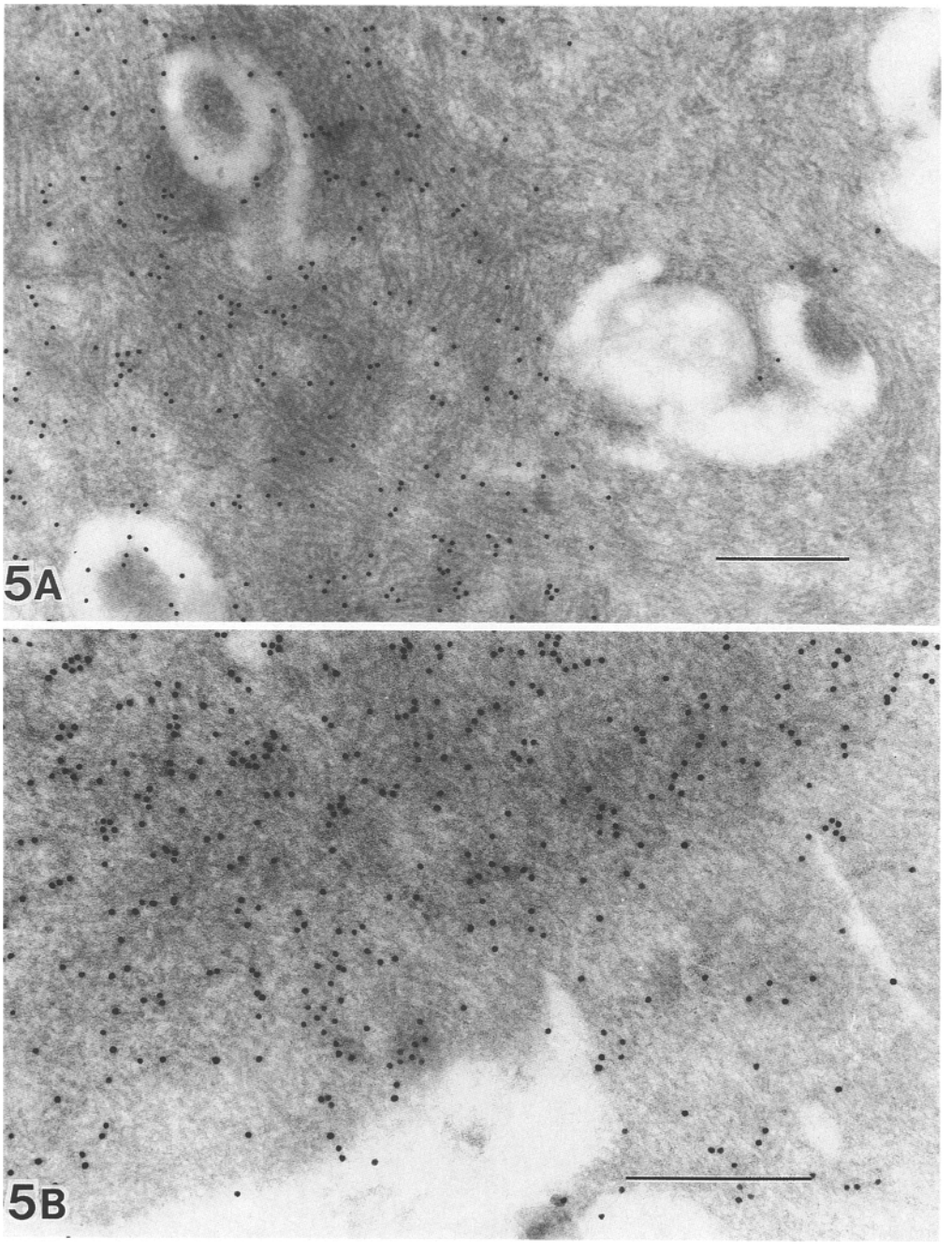

Fig. 5A, B Electron micrographs illustrating immunocytochemical labelling of microtubules in osteoclast cytoplasm with anti-respiratory syncytial virus (**A**) and anti-measles virus (**B**) antibody-colloidal gold conjugates. Bar = 0.5 μm. × 30,000 (**A**) and × 40,000 (**B**)

action of two "incomplete slow viruses", neither of which is able to cause the disease itself, or that there exists a single RNA mutant virus that contains antigenic determinants to both (Mirra 1987). It is possible that both viruses are present but defective, and one may serve as a helper virus pointed by Mills and Singer (1976). Our study is the first to demonstrate binding of anti-virus antibodies to virus type inclusion bodies at the electron microscope level. It is difficult to evaluate the significance of the positive or negative reaction to the virus antibody we have observed and application of our method to many more cases should provide a key to this problem.

References

Baslé MF, Fournier JG, Rozenblatt S, Rebel A, Bouteille M (1986) Measles virus RNA detected in Paget's disease bone tissue by in situ hybridization. J Gen Virol 67:907–909

Baslé MF, Rebel A, Fournier JG, Russell WC, Malkani K (1987) On the trial of paramyxoviruses in Paget's disease of bone. Clin Orthop 217:9–15

Gherardi G, LoCascio V, Bonucci E (1980) Fine structure of nuclei and cytoplasm of osteoclasts in Paget's disease of bone. Histopathologie 4:63–74

Greenspan A (1991) A review of Paget's disease: radiologic imaging, differential diagnosis and treatment. Bull Hosp Jt Dis Orthop Inst 51:22–33

Gyorkey F, Sinkovics JG, Gyorkey P (1972) Electron microscopic observations on structures resembling myxovirus in human sarcomas. Cancer 27:1449–1454

Harvey L, Gray T, Beneton MN, Douglas DL, Kanis JA, Russell RGG (1982) Ultrastructural features of the osteoclasts from Paget's disease of bone in relation to a viral aetiology. J Clin Pathol 35:771–779

Hino M, Aoki J, Shigeno C, Yamamoto I, Torizuka K, Higa T (1986) Histological findings of Paget's disease of bone. Jap J Bone Metab 4:100–108.24

Howatson AF, Fornasier VL (1982) Microfilaments associated with Paget's disease of bone: comparison with nucleocapsids of measles virus and respiratory syncytial virus. Intervirology 18:150–159

Leduc EH, Marinozzi V, Bernhard W (1963) The use of water-soluble glycol methacrylate in ultrastructural cytochemistry. J R Microsc Soc 81:119–130

Merkow RL, Lane JM (1990) Paget's disease of bone. Orthop Clin North Am 21:171–189

Mii Y, Miyauchi Y, Kambara K, Honoki K, Masuhara K (1988) Intracytoplasmic crystalloid structures in a malignant fibrous histiocytoma: ultrastructural and cytochemical study. Ultrastruct Pathol 12:537–546

Milgram JW (1977) Radiographical and pathological assessment of the activity of Paget's disease of bone. Clin Orthop 127:43–54

Mills BG, Singer FR (1976) Nuclear inclusions in Paget's disease of bone. Science 194:201

Mills BG, Singer FR (1987) Critical evaluation of viral antigen data in Paget's disease of bone. Clin Orthop 217:16–25

Mills BG, Singer FR, Weiner LP, Holst PA (1980) Cell cultures from bone affected by Paget's disease. Arthritis Rheum 23:1115–1120

Mills BG, Singer FR, Weiner LP, Holst PA (1981) Immunohistological demonstration of respiratory syncytial viral antigens in Paget's disease of bone. Proc Natl Acad Sci USA 78:1209–1213

Mirra JM (1987) Pathogenesis of Paget's disease based on viral etiology. Clin Orthop 217:162–170

Mizuhira V (1990) New fixation method by the aid of microwave energy irradiation – fixation method and its theoretical background. Electron Microsc 25:44–57

Paget J (1877) On a form of chronic inflammation of bones (oesteitis deformans). Trans R Med Chir Soc Lond 60:37–63

Rebel A, Malkani K, Baslé MF, Bregeon Ch (1974) Particularites ultrastructurales des osteoclastes de la maladie de Paget. Rev Rhum Mal Osteoartic 41:767–771

Rebel A, Basle M, Pouplard A, Malkani K, Filmon R, Lepatezour A (1980) Bone tissue in Paget's disease of bone. Ultrastructure and immunocytology. Arthritis Rheum 23:1104–1114

Singer FR (1980) Paget's disease of bone: a slow virus infection? Calcif Tiss Int 31:185–187

Siris ES, Canfield RE (1982) Paget's disease of bone. Current concepts as to its nature and management. Orthop Review 11:43–49

Siris ES, Ottman R, Flaster E, Kelsey JL (1991) Familial aggregation of Paget's disease of bone. J Bone Miner Res 6:495–500

Sissons HA (1966) Epidemiology of Paget's disease. Clin Orthop 45:73–79

Sofaer JA, Holloway SM, Emery AEH (1983) A family study of Paget's disease of bone. J Epidemiol Commun Health 37:226–231

Takashima T, Kawai K, Miki A, Ukai K, Hirohata K (1988) Paget's disease of bone – inclusion bodies in the osteoclasts. J Clin Electron Microsc 21:5–6

Timms BG (1986) Postembedding immunogold labeling for electron microscopy using LR White resin. Am J Anat 175:267–275