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Paramyxovirus Antigens in Osteoclasts from Paget's Bone Tissue Detected by Monoclonal Antibodies

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SUMMARY

The fluorescent antibody technique using both monoclonal and specific polyclonal virus antibodies was applied to investigate the nature of the inclusions seen in the abnormal osteoclasts associated with Paget's bone disease. The results show that antigens of measles virus, simian virus 5 (SV5) and human parainfluenza virus type 3 (PF3) could be detected in the osteoclasts but not in control bone cells. Measles and SV5 nucleoprotein (NP) and haemagglutinin-neuraminidase (HN) antigens were apparently present in all the cases of Paget's disease examined, whereas PF3 NP and HN antigens were present only in some of the cases. These investigations suggest that paramyxoviruses may play a role in the aetiology of the bone disease.

INTRODUCTION

Paget's bone disease is usually a benign disorder of a chronic nature. The disease mainly affects subjects over 50, the frequency increasing with age. Histologically, it is characterized by considerable disorganization of the structure of bone trabeculae, by the presence of large quantities of fibrotic tissue in the medullary spaces where the bone marrow disappears, and by very active bone remodelling with huge multinucleated osteoclasts leading to anarchic bone resorption (Hamdy, 1981).

The aetiology of Paget's bone disease has led to much discussion and since the hypothesis put forward by Paget himself in 1877 that it might be caused by inflammatory disorders, various other theories have been proposed, e.g. endocrine disfunction, inherited anomalies of connective tissue, or autoimmune phenomena (Hamdy, 1981). Since 1974, several studies have suggested that the disease may be of virus origin. Thus, it has been demonstrated that in Paget's bone disease the characteristic giant osteoclasts, presumed to arise from the fusion of cells (probably of monocyte origin) contain microcylindrical inclusions whose structure, dimensions and intracellular location are all very similar to those of paramyxovirus nucleocapsids in infected host cells (Gherardi et al., 1980; Harvey et al., 1982; Mills & Singer, 1976; Rebel et al., 1974). Immunological studies using polyclonal antisera have since revealed that the osteoclasts in Paget's bone disease contain viral antigens of the paramyxovirus group, both measles virus (Basle et al., 1979; Rebel et al., 1980 a; Mills et al., 1982) and respiratory syncytial virus (Mills et al., 1982, 1984; Singer & Mills, 1983) having been detected.

In this study we have applied fluorescent antibody techniques to bone samples from patients with Paget's disease as well as from controls using well defined monoclonal or monospecific antibodies directed against different viruses and in particular against viruses of the paramyxovirus group.
METHODS

Bone samples. The bone samples were obtained from six untreated patients known to have Paget's disease, by performing transiliac bone biopsies in zones affected by the disease as identified by X-ray analysis. Control bone samples were obtained from three patients with hyperparathyroidism, bone fluorosis or extensive bone remodelling after traumatic fracture, all samples being rich in osteoclasts. Bone biopsies were rapidly fixed by total immersion for 90 min in 4% formaldehyde phosphate-buffered to pH 7.4. Bone fragments, 1 to 2 mm$^3$, were decalcified with 0.1 M EDTA phosphate-buffered to pH 7.4, at 4 °C for 90 min (3 × 30 min) and then embedded in paraffin wax.

Fluorescent antibody technique. Five μm thick sections were cut and picked up on pretreated gelatin–albumin-coated slides. After drying at 45 °C for 48 h the slides were deparaffined by three washes in xylene and progressively rehydrated. Fifty μl drops of monoclonal or monospecific antibodies, diluted in phosphate buffer (1/25, 1/50, 1/100, 1/200 dilutions) were deposited upon the deparaffined and rehydrated bone sections and incubated for 90 min at 20 °C in a humidifier. After rinsing three times in phosphate buffer (10 min, 60 min and 10 min), 50 μl of an appropriate dilution of fluorescein isothiocyanate-conjugated goat anti-mouse globulin or anti-bovine globulin (GAM–FITC, GAB–FITC; Nordic Immunological Laboratories) was added and the slides were incubated again for 45 min as described above. The slides were rinsed again three times in phosphate buffer, mounted and analysed by standard fluorescence microscopy.

Antibodies. Several types of antibodies directed against different viral antigens were tested in a double-blind fashion. Monoclonal antibodies as ascitic fluids against simian virus 5 (SV5) nucleoprotein and haemagglutinin–neuraminidase protein (NP, HN) (Goswami & Russell, 1983), parainfluenza virus type 3 (PF3) NP and HN antigens (Goswami & Russell, 1983), measles virus nucleoprotein (NP), haemagglutinin (HA), fusion protein (F) and membrane protein (M) (Giraudeau et al., 1984; Giraudeau & Wild, 1981; Norbury et al., 1982; Russell & Goswami, 1984; Togashi et al., 1981), respiratory syncytial virus (RSV) nucleoprotein (NP-1, NP-2) [sera supplied by Dr P. J. Watt (Department of Microbiology, University of Southampton Medical School, Southampton, U.K.)], mumps virus antigens, influenza virus NP [Russell & Goswami, 1984], and human adenovirus type 5 hexon (Russell et al., 1981) were used. Polyclonal anti-human RSV, anti-bovine RSV and anti-human PF3 virus monospecific sera [supplied by Dr E. J. Stott (National Institute for Research on Animal Diseases, Compton, U.K.)] were also tested. They were raised in gnotobiotic calves, born and bred in germ-free conditions.

Cell cultures. Monkey kidney Vero cell cultures, uninfected or infected by various strains of measles virus (LEC, Hallé or Edmonston) were used to check antibody specificity and to define the optimum dilutions of the measles antibodies. The culture medium was RPMI 1640 (Gibco) enriched with 2% foetal calf serum. After 24 h of infection, the first cytopathic effects appeared in the form of syncytia. At this point the cells were abundantly washed with phosphate buffer pH 7.4, fixed in 2% formaldehyde for 5 min and treated with 0.1 M EDTA for 3 min before being analysed.

RESULTS

Six bone tissue samples from Paget’s bone disease and three control bone tissue samples were tested by the indirect immunofluorescence technique using monoclonal antibodies and monospecific antisera against measles, SV5, PF3, mumps, RSV, influenza and adenovirus antigens. The results obtained are summarized in Table 1. The positivity of a reaction was judged either by the presence of round or oval fluorescent spots of various sizes in the cytoplasm and/or nuclei of the cells or else by the presence of a finely granular and diffuse cytoplasmic fluorescence.

The positive reactions obtained with monoclonal antibodies on diseased bone tissue were characterized by specific fluorescence uniquely in osteoclasts. The other cells of the bone tissue and the medullary spaces remained negative.

The monoclonal antibodies against the different measles virus antigens (NP, M, F, HA) all gave positive reactions, each antibody locating antigens within the osteoclastic giant cells at characteristic locations, e.g. anti-F antibody detected discrete granules mostly in the cytoplasm whereas anti-NP detected antigens as a more diffuse slightly granular fluorescence in both the nuclei and cytoplasm (Fig. 1). All the six bone tissue samples derived from Paget’s patients gave positive results (Table 1) at antibody dilutions up to 1 in 200. Higher dilutions gave diminished fluorescence consistent with the behaviour of the antibodies when tested on infected cells fixed by a similar procedure to that used for the bone sections.

Positive results were also obtained with monoclonal antibodies directed against the viral NPs and HN glycoprotein of SV5 and PF3 (Fig. 2). With these antibodies, specific fluorescence was
localized in osteoclasts in the form of intracytoplasmic granules. The results in Table 1 show that the positive score (6/6) obtained with anti-SV5 was similar to that with anti-measles monoclonal antibodies although the SV5 HN antibody showed greatly reduced fluorescence at higher dilutions where the antigen could still be detected in infected cells (Goswami & Russell, 1983). Using anti-PF3 monoclonal antibodies we found some positive results (3/6) with anti-NP but only one positive result with anti-HN at the 1/50 dilution, i.e. at dilutions significantly lower than those showing positive reactions in virus-infected cells (Goswami & Russell, 1983).

With all the other types of antibodies, whether monoclonal (anti-RSV NP, anti-mumps, anti-influenza NP and anti-adenovirus type 5) or monospecific polyclonal (anti-bovine RSV, anti-human RSV, anti-bovine PF3), the results were always negative in our tests even at high antibody concentrations (Table 1).

In control experiments with antibodies directed against the structural proteins of measles
Table 1. *Immunofluorescence tests with monoclonal antibodies directed against paramyxoviruses*

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<tr>
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<th>Measles</th>
<th>SV5</th>
<th>PF3</th>
<th>RSV</th>
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<td>Antibody dilution</td>
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<td>Fluorescence in Paget osteoclasts</td>
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<td>Proportion of positive results</td>
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<td>6/6</td>
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<td>Fluorescence in control osteoclasts</td>
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* No fluorescence was noted using specific polyclonal antibodies against RSV and PF3 viruses even using dilution of 1/25.
virus (NP, HA, F, M), the immunofluorescence reactions were positive on cells infected with different measles strains (LEC, Hallé or Edmonston). With all the other antibodies no positive reaction was observed on measles-infected Vero cells or uninfected cells, consistent with the properties of the antibodies found in previous studies [e.g. these measles antibodies did not show cross-reactions with uninfected cells as described by Fujinami et al. (1983) (see Lane & Koprowski, 1982; Nigg et al., 1982)].

Experiments carried out on control bone samples, from patients free of Paget's bone disease, never showed positive results for any of the monoclonal antibodies or polyclonal monospecific sera used (Fig. 2c).

Fig. 2. Fluorescence photomicrographs of bone tissue sections from a patient with Paget's bone disease stained with antibodies to (a) SV5 nucleoprotein, (b) PF3 nucleoprotein. Specific fluorescence was seen only in the multinucleated osteoclasts. (c) Control bone tissue section from a patient with bone fluorosis, fluorescently stained with anti-measles NP monoclonal antibody at 1/25 dilution. No specific fluorescence was observed: compare with Fig. 1(a). Magnification × 750.
DISCUSSION

The results obtained in this study show, rather surprisingly, that osteoclasts in Paget's bone disease tissue contain antigens reacting with monoclonal antibodies against the major proteins of measles and SV5 viruses. More limited reactions were obtained with monoclonal antibodies against PF3 in some of the patients. It seems reasonable to assume that these antigens are related to the presence of the characteristic microcylindrical inclusions noted in the diseased osteoclasts although immunoelectron microscopic techniques will be necessary to verify this conjecture.

The observations made with monoclonal antibodies directed against measles virus proteins thus confirm previous results obtained with measles polyclonal sera (Basle et al., 1979; Rebel et al., 1980a, b; Mills et al., 1982). It is interesting that the intracellular distribution of measles antigens in the osteoclasts (Fig. 1) is more consistent with the pattern found in persistently infected tissue culture cells (Giraudon et al., 1984; Norrby et al., 1982). Thus the measles NP antigens could be detected in both nuclei and cytoplasm and indeed agree with ultrastructural studies which show that both the cytoplasm and nuclei of Pagetic osteoclasts contain microcylindrical structures very similar in dimensions and nature to paramyxovirus nucleocapsids (Rebel et al., 1980a, b). It also seems very unlikely that these positive reactions arise from epitopes that cross-react with activated bone antigens since all the different measles and SV5 monoclonal antibodies react in the tests.

The presence of SV5 and PF3 antigens in Pagetic osteoclasts has never been reported before but it is consistent with observations that antigens of both of these viruses can be demonstrated in human bone marrow cells, indicating persistent (possibly defective) infection (Goswami et al., 1984a). Moreover, using specific SV5 monoclonal antibodies it is apparent that SV5 infects a significant proportion of humans (Goswami et al., 1984b). The relationship between haematogenous bone marrows and bone tissues is not very clear but current theories suggest that a multipotent stem cell, perhaps an endosteal cell lying at the trabecular bone surface, is common to all haematogenous cell lines and to bone cells (Duheille et al., 1980; Schofield, 1978; Sultan, 1980). Moreover, the osteoclast, which apparently originates in bone marrow probably via a mononuclear cell (Hanakoa, 1979; Horton et al., 1984; Severson, 1983), seems to be the abnormal cell in Paget's bone disease. It should also be borne in mind that osteoclasts appear to have some characteristics akin to macrophages, perhaps even the property of bone resorption through phagocytic mechanisms (Chambers, 1980; Marks, 1983; Mundy, 1983). It may therefore be that the scavenging activities of these cells account for the presence of the different virus antigens. Thus, one could speculate that the viruses may be present in other cells as a persistent infection and have been released and sequestered by Pagetic osteoclasts following derepression by some process (e.g. loss of immune control). It is, of course, impossible at this stage to decide whether the presence of the virus antigens is the primary cause of the disease or is a secondary phenomenon.

The antibodies directed against RSV consistently gave negative results on the Pagetic osteoclasts in our tests using either monoclonal or more specific polyclonal antibodies, contrary to published reports (Mills et al., 1982; Singer & Mills, 1983). Although we have used polyclonal sera generously supplied by Dr F. R. Singer we have not been able to obtain unambiguous positive fluorescence on our bone samples. It may be that variations in the processing and fixing of the bone tissues in the two laboratories could account for at least some of the differences found, since it seems unlikely that RSV, which causes infections world-wide, could be present only in bone tissue of American origin.

There are also some minor inconsistencies with respect to fluorescent staining with the human PF3 reagents: the monoclonal PF3 antibodies appear to react with some of the Pagetic bone, whereas the bovine polyclonal serum does not. This may reflect differences in the relative sensitivities of the antibodies or of the second (fluorescein-conjugated) antibodies, but no attempts were made to assess these differences.

These studies with highly specific reagents therefore confirm and expand the previous observations and clearly show that some viruses belonging to the Paramyxoviridae can persist in human tissues and may play an aetiological role in Paget's bone disease.
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