Measles Virus RNA Detected in Paget's Disease Bone Tissue by in situ Hybridization

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SUMMARY

Morphological and immunocytological studies have demonstrated the presence of paramyxovirus antigens in Paget's bone disease tissue and in particular antigens related to measles virus and respiratory syncytial virus. To examine the relationship between measles virus and Paget's bone disease we used in situ hybridization and a cloned measles virus DNA probe specific for the nucleocapsid protein to detect and locate measles virus RNA sequences in Paget's bone tissue. In five patients with the disease, measles virus RNA sequences were detected not only in 80 to 90% of the multinucleated osteoclasts where there is morphological and immunocytological evidence of measles virus activity but also in 30 to 40% of mononucleated bone cells, mainly osteoblasts, osteocytes, fibroblasts and lympho-monocytes. In contrast, no hybridization was observed in bone tissue from three control patients without signs of Paget's bone disease. These results indicate that the host cell range for measles virus in Paget's disease is more widespread than has been supposed. They also demonstrate the usefulness of the in situ hybridization method to detect viral genetic information in cells where viral antigenic activity is not detectable. These observations further support the hypothesis that measles virus is involved in the pathogenesis of Paget's bone disease.

INTRODUCTION

Osteitis deformans, first described by Paget in 1876, is a chronic human bone disorder. The frequency increases with age and affects about 4% of the population over 70 years of age. The disease is often asymptomatic but is sometimes associated with painful, deformed bones, fractures, nerve compression due to hypertrophied bone and, in some cases, with sarcomatous degeneration. Histological studies have revealed that bone tissue from patients with Paget's disease has an abnormally irregular structure with considerably increased cellular activity, both of osteoclasts and osteoblasts, together with invasion of the medullary space by fibrotic tissue which gradually replaces the hematogenous marrow (Hamdy, 1981; Krane, 1977).

Although the aetiology of Paget's bone disease still remains to be established, electron microscopical and immunocytological observations suggest that it could be linked to a paramyxovirus infection. Ultrastructural studies have shown the presence of specific inclusions in the enormous, multinucleated osteoclasts from Pagetic bone tissue. Morphological analyses have demonstrated that the structure, the dimensions and the cytoplasmic and nuclear distribution of the microcylindrical inclusions observed in Pagetic osteoclasts are very similar to the nucleocapsids found in cells infected with paramyxoviruses (Gherardi et al., 1980; Harvey et al., 1982; Howatson & Fornasier, 1982; Mills & Singer, 1976; Rebel et al., 1980b, 1974). Furthermore, using immunocytological techniques with polyclonal antibodies, several studies have demonstrated that osteoclasts from bone tissue in Paget's disease contain antigenic material related to measles virus and to respiratory syncytial virus, both of which are members of
the family *Paramyxoviridae* (Baslé et al., 1979; Mills et al., 1982; Rebel et al., 1980a; Singer & Mills, 1983). The results we obtained using monoclonal antibodies suggest that a number of different paramyxoviruses may be involved in the disease and we have also confirmed the presence of the main structural proteins of measles virus in osteoclasts from Paget's bone tissue, namely the nucleocapsid, haemagglutinin–neuraminidase, fusion and membrane proteins (Baslé et al., 1985). The significance of these results is not yet clearly established. The role of measles virus, respiratory syncytial virus or of another type of virus in the pathogenesis of Paget's disease is still under discussion (Baslé et al., 1979, 1985; Mills et al., 1982, 1985).

In order to analyse further the relationship of measles virus and Paget's bone disease, we have studied the presence and the localization of measles virus RNA in bone tissue from patients with the disease and from control subjects. In this study, we have used the *in situ* hybridization technique and a cloned DNA specific for measles nucleocapsid protein to establish whether measles virus nucleic acid sequences were present in multinucleated osteoclasts and whether they could also be observed in other types of cell in Paget's bone tissue.

**METHODS**

*Preparation of bone tissue samples.* Bone tissue samples were obtained from five patients with Paget's bone disease by transiliac bone biopsy carried out in zones affected by Paget's disease as identified by X-ray analysis. Bone fragments about 1 to 2 mm\(^3\) were randomly fixed by total immersion for 90 min in 2% formaldehyde phosphate-buffered to pH 7.4, decalcified for 2 weeks at 4 °C in 0.1 M-EDTA in phosphate buffer pH 7.4 with constant agitation, rinsed several times in phosphate buffer and then embedded in paraffin. Sections 5 μm thick were cut from tissue blocks and picked up on pretreated gelatin–albumin-coated slides. The slides were dried at 45 °C for 48 h, deparaffined by three washes in xylene, and rinsed in 100% ethanol for 5 min. To enhance the diffusion of the probe, rehydrated bone sections were treated for 10 min in 0.2 M-HCl, followed after a brief washing in distilled water by 15 min at 37 °C in 20 mM-Tris-HCl pH 7.4, 2 mM-CaCl\(_2\), containing 15 μg/ml proteinase K. Finally the slides were dehydrated in graded ethanol and vacuum-dried.

Control bone samples were obtained from three patients with bone fluorosis, fracture bone healing and hyperparathyroidism. Bone tissue controls were treated and tested as described for Paget's bone tissue.

*Probes.* Plasmid pBR322 containing cDNA specific for the measles virus nucleocapsid protein was used as a probe and was prepared from the poly(A) mRNA extracted from the CV-1 line of African green monkey kidney cells infected with measles virus as previously described (Gorecki & Rozenblatt, 1980). The insert of cloned cDNA contains 1420 base pairs and the size of the RNA complementary to this cloned cDNA is 1750 nucleotides (Gorecki & Rozenblatt, 1980). A pBR322 vector containing hepatitis B virus (HBV) DNA and pBR322 alone were used as controls (Dubois et al., 1980; Fournier et al., 1982a, b).

The viral DNA probes were tritiated by nick translation (Maniatis et al., 1975). The reaction mixture used contained four deoxynucleoside triphosphates: \([\text{H}]\text{dCTP diluted to 50 Ci/mmol}, [\text{H}]\text{dTTP diluted to 100 Ci/mmol}, [\text{H}]\text{dGTP diluted to 7 Ci/mmol and [H]}\text{dATP diluted to 24 Ci/mmol (Amersham).}\]

The unincorporated nucleotides were separated from the labelled DNA by filtration on a Sephadex G-50 (Pharmacia) spin column for 4 min. The DNA was co-precipitated with 2 mg/ml of salmon sperm DNA in ethanol, dried and redissolved in distilled water. The specific activity obtained was around 2.5 × 10\(^7\) c.p.m./μg.

*In situ hybridization procedures.* For the detection of intracellular viral nucleic acid we used *in situ* hybridization procedures as previously described but with minor modifications (Brahic & Haase, 1978; Fournier et al., 1983). Each slide was treated with 20 μl of a solution containing 50% (v/v) freshly deionized formamide, 10 mM-Tris–HCl pH 7–4, 1 mM-EDTA, 600 mM-NaCl, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin, 10 mg/ml CV-1 cell RNA and 5 ng 3H-labelled DNA previously denatured at 100 °C for 3 min. Hybridization was carried out under coverslips at 37 °C for 24 h. After four washes (15 min each time) in 0.3 M-NaCl, 0.03 M-sodium citrate pH 7.0, the slides were washed overnight at room temperature in the same buffer. They were then dehydrated in graded ethanol (70% twice, 95% once, 100% twice; 3 min each time) and air-dried. As an additional control, hybridization was also carried out after treatment of the slide with 100 μg/ml RNase for 1 h at 37 °C.

*Autoradiography.* The slides were coated with K5 Ilford emulsion diluted 1:1 in distilled water, air-dried again at room temperature and stored in a dark-room. The slides were exposed for 3 weeks at 4 °C, developed in Kodak D9 diluted 1:1 in distilled water at room temperature and stained with haematoxylin and eosin (Gabe, 1968).

**RESULTS**

The preparation of bone samples from patients with Paget's disease as described above allowed excellent preservation of tissue and cell morphology as well as the detection of hybridizable measles virus RNA molecules.
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Fig. 1. Demonstration by in situ hybridization of measles virus nucleic acid sequences in Paget's bone tissue. (a) Multinucleated osteoclast (OC) with cytoplasmic expansion at the endosteal surface (arrows) (×900); (b) osteoblasts (OB) (×810); (c) osteocytes (×900); (d) fibroblasts (×990). All the bone sections were hybridized for the same time in the same batch. Autoradiographic grains are widely distributed over the osteoclast both in cytoplasm and in nuclei. In mononucleated cells (osteoblasts, osteocytes, fibroblasts) the labelled probe is mainly detected over the cytoplasmic area. Exposure 3 weeks; haematoxylin. BM, Decalcified bone matrix.

The haematoxylin and eosin stain identifies the different bone cells and reveals intercellular relationships as well as relations between bone cells and the trabecular endosteal surfaces (Hamdy, 1981; Krane, 1977; Rebel et al., 1980b). Osteoclasts are voluminous with an unusually large number of nuclei. These cells are very often found lying against bone surfaces within resorption cavities. Osteoblasts are mononucleated, prism-shaped cells arranged in pseudo-epithelial formation at the endosteal surface. Osteocytes, also mononucleated, are found in osteocytic lacunae within bone tissue. Endosteal cells are seen flattened against bone trabecular surfaces. Cell identification is rather more difficult in the medullary spaces. However, clusters of mononucleated cells, lymphocytes and monocytes, and a few isolated cells, probably fibroblasts, as well as capillary cells are easily identified.

By in situ hybridization, measles virus RNA sequences were detected in several types of cell in Paget's bone tissue. Results were similar for the five cases of Paget's bone disease tested, and the labelling appeared to be specific because it could be traced on serial sections through the same cells and had similar localizations of the silver grains. Osteoclasts were the most frequently involved type of cell (Fig. 1a). In 80 to 90% of these multinucleated cells, hybridization reactions
Fig. 2. *In situ* hybridization reactions. (a) In Paget's bone tissue, measles virus nucleic acid sequences are demonstrated in clusters of lymphocytes and monocytes, and the labelling is mainly detected over the cytoplasmic area (×900). (b) Pretreatment of Paget's bone tissue with 100 μg/ml RNase (1 h; 37 °C) followed by *in situ* hybridization with the measles virus ³H-labelled probe reduces the signal to background level (×900). (c) Paget's bone tissue shows no hybridization reaction with ³H-labelled HBV DNA probe. Compare the osteoclast seen here with the osteoclast in Fig. 1(a) (×1080). (d) No hybridization is observed in control bone tissue with ³H-labelled measles virus DNA probe (×1080). Exposure 3 weeks; haematoxylin. BM, Decalcified bone matrix.

were observed both in the cytoplasm, with a diffuse distribution, and in some of the numerous nuclei, with different levels of intensity. Autoradiographic grains were sometimes concentrated and localized over the nuclear area, in particular over clear nuclear inclusions which very probably correspond to the nuclear accumulation of specific microcytinders as previously described in Pagetic osteoclasts (Gherardi *et al.*, 1980; Harvey *et al.*, 1982; Mills & Singer, 1976; Rebel *et al.*, 1980b, 1974).

Between 30 and 40% of the mononucleated cells in Paget's bone tissue were found to hybridize with the ³H-labelled DNA probe specific for measles virus RNA but it was not possible to obtain a specific count for each type of cell. Endosteal cells, osteoblasts, osteocytes and fibroblasts were occasionally labelled, mainly over the cytoplasmic surface (Fig. 1b, c, d). In addition, the clusters of mononucleated cells present in the medullary spaces showed many
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positive cells. In most of these cells, autoradiographic grains were observed predominantly over the cytoplasmic area. However, accumulations of silver grains were also observed over a few nuclei (Fig. 2a).

The specificity of the assay for RNA was confirmed by the negative result obtained by pretreatment of bone tissue sections with 100 μg/ml RNase for 1 h at 37 °C; this reduced the hybridization signal to background levels (Fig. 2b). The hybridization observed in Paget's bone tissue was specific for measles virus nucleotide sequences since the use of unrelated tritiated probes (HBV, pBR322) did not result in hybrid formation (Fig. 2c).

Finally, bone tissue from control patients showed no hybridization reaction with any of the labelled probes (Fig. 2d).

DISCUSSION

The presence of measles virus RNA sequences was detected in cells from bone tissue in Paget's disease by in situ hybridization with a labelled DNA probe containing specific sequences for the nucleocapsid protein. From a methodological point of view, the standard technique had to be modified to enable study of bone tissue. We used formaldehyde at 2% for fixing our samples as this medium penetrates bone tissue rapidly, without dehydration, and produces no crosslinks. Bone tissue is heterogeneous with a highly mineralized collagen matrix and large medullary spaces containing haematogenous marrow and for this reason we preferred paraffin embedding to methods involving freeze microtomy. Consequently, the bone samples had to be decalcified prior to ordinary histological observation. Partial deproteinization, with HCl or proteinase K, allowed the probe to diffuse more freely and gain easier access to intracellular ribonucleic acid molecules. With these modifications we obtained a highly efficient hybridization reaction with excellent preservation of cell morphology. A similar approach was previously used to detect HBV DNA with a specific probe, in liver cells from a patient with chronic active hepatitis B, cirrhosis and hepatocellular carcinoma (Blum et al., 1984).

The cloned measles virus DNA probe used here is specific for measles nucleocapsid RNA (Fournier et al., 1983; Gorecki & Rozenblatt, 1980). With this probe, hybridization reactions were found in various types of cell in bone tissue samples from cases of Paget's disease, with a similar aspect in all five cases examined but with different patterns in the distribution of the silver grains according to cell type.

Measles virus RNA was detected in 80 to 90% of the multinucleated osteoclasts, both in cytoplasm and in nuclei. This double localization of measles virus RNA in the cell is most likely associated with transcription and replication processes of the virus as was observed in an in vitro infection (Fournier et al., 1983). These findings are consistent with the ultrastructural and immunocytological observations previously described. Electron microscopical studies have shown that the cytoplasm and nuclei of osteoclasts from Paget's bone tissue contain microcylindrical inclusions very similar to measles virus nucleocapsids (Gherardi et al., 1980; Harvey et al., 1982; Howatson & Fornasier, 1982; Rebel et al., 1980b). Immunological experiments have also demonstrated the presence of measles nucleocapsid protein antigens both in cytoplasm and in nuclei of Pagetic osteoclasts (Baslé et al., 1979, 1985; Rebel et al., 1980a). It is now well known that measles virus RNA is closely associated with the nucleocapsid both in the cytoplasm and nuclei of infected cells (Fraser & Martin, 1978; Rentier, 1981). Our study demonstrates that osteoclasts from Paget's bone tissue contain RNA sequences specific for measles nucleocapsid protein.

Measles virus RNA sequences are also detectable in 30 to 40% of mononucleated cells in Paget's bone tissue. However, it is of interest to note that in most of these mononucleated cells, the hybridization sites are predominantly in the cytoplasm. Only 10 to 20% of the labelled mononucleated cells show cytoplasmic and nuclear labelling. In osteoblasts and osteocytes the silver grains are scattered over the cytoplasmic and nuclear areas of the cells. In some fibroblasts, hybridizable molecules were concentrated mainly in the cytoplasm and in the lympho-monocyte clusters the labelling is predominantly located in the cytoplasm with few, if any, silver grains over the nuclear area. These results are rather unexpected for mononucleated
cells in which ultrastructural observations have never shown nucleocapsid formations and in which immunocytological studies have failed to demonstrate any viral antigen. It therefore appears that measles viral RNA is present in these cells but does not result in detectable antigen. Under our experimental conditions it was not possible to distinguish between the genomic and the messenger RNA, which are similarly detectable by the double-stranded cloned DNA probe we used in our experiments.

An identical situation was observed in peripheral blood lymphocytes of patients with subacute sclerosing panencephalitis (Fournier et al., 1985). In this slow neurological disease, closely linked with a persistent measles virus infection, nerve cells have been shown to contain paramyxovirus nucleocapsids but brain and circulating lymphocytes failed to express detectable viral antigens (Fournier et al., 1985). In situ hybridization carried out with the same probe used in the present study, however, demonstrated the presence of measles virus RNA sequences in neurons and glial cells in brain parenchyma. Furthermore, measles virus RNA molecules were also detected in peripheral blood lymphocytes and in infiltrating cells of the perivascular cuffs consisting mainly of lymphocytes (Fournier et al., 1985). Similar observations were also reported concerning a widespread distribution of HBV DNA in hepatocytes, bile duct epithelium and vascular elements in HBV-infected liver (Blum et al., 1983). Thus, in situ hybridization appears to be a more effective method to demonstrate viral infection than morphological or immunocytological techniques.

The detection of measles virus RNA in several types of cell in bone tissue from Paget's disease indicates that the host cell range of measles virus is more extensive than has been found by morphological and immunocytological criteria. The presence of measles virus RNA in cells from Paget's bone tissue does not, by itself, necessarily incriminate this virus in the disease. However, the huge and multinucleated osteoclasts from Paget's bone tissue resemble the polykaryons observed in measles virus-infected cell cultures and could correspond to the cytopathogenic effect of this virus.

These findings also demonstrate the usefulness of in situ hybridization in revealing viral genetic information not only in infected cells where there are morphological disturbances or expression of immunological markers, but also in cells not suspected of containing viral genetic information.

A similar approach should be attempted to look for RNAs of other paramyxoviruses, and especially for those of respiratory syncytial virus, in Paget's bone tissue as soon as specific probes are available. The demonstration that measles virus RNA is present in cells from diseased tissue further supports the proposition that a paramyxovirus may be linked to Paget's disease of bone.

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