Ultrastructural features of the osteoclasts from Paget's disease of bone in relation to a viral aetiology

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SUMMARY The ultrastructure of the osteocytes, osteoblasts, osteoclasts, haemopoietic and other connective tissue cells was examined in 27 biopsies from 22 patients with Paget's disease of bone. Electron microscopy showed characteristic nuclear and cytoplasmic inclusions in the osteoclasts of all of the 25 biopsies exhibiting histological evidence of Paget's disease. Such inclusions were absent from all the other types examined. The intranuclear inclusions consisted of stacked rows or complex whorls of tubular filaments with an individual filament diameter of 12-15 nm, often arranged in a paracrystalline array. The frequency of occurrence of inclusions in the osteoclasts and their individual nuclei measured quantitatively in 18 of the biopsies was related to the histological severity of the disease process. The similarity of the observed inclusions to those of paramyxovirus inclusion bodies (particularly measles) support the hypothesis that Paget's disease is a slow virus infection.

Paget's disease of bone has a highly variable geographical incidence.¹ In parts of Europe it affects 3-4% of the population over the age of 40.² The aetiology of this relatively common disorder remains unknown. Suggested causes range from Paget's own theory of chronic inflammation³ to that of a slowly progressive benign neoplasm (see Singer and Mills⁴ for a review). The characteristic giant osteoclasts seen in the early phases of the disease,^{5,6} not seen in other bone conditions, has led to the view that Paget's disease is primarily due to osteoclast dysfunction. Indeed ultrastructural abnormalities in osteoclasts first reported by a group of French workers,⁷ led them to suggest a slow viral infection as a possible aetiology.⁸ There is, however, some controversy concerning both the nature of the inclusion bodies and their specificity for osteoclasts, suggesting that fixation and processing artefacts may complicate such investigations. These problems stimulated us to investigate the ultrastructural abnormalities in our patients with Paget's disease.

Materials and methods

Twenty-seven biopsies were examined from 22 patients aged 44-86 y. All had radiographic,

biochemical and clinical evidence of Paget's disease. Twenty-five of the 27 biopsies had histological evidence of disease, with increased numbers of giant osteoclasts, increased proportions of trabecular bone surfaces occupied by osteoblasts as well as marrow fibrosis and a mosaic pattern in trabecular bone (Fig. 1). Two biopsies showed normal bone: Osteoclasts were not seen but all the other bone cell types were noted. Twenty-four of the biopsies were iliac trephine biopsies varying from 4-7 mm in diameter and 5-10 mm in length. The other three were a necropsy specimen of skull and open surgical biopsies of humerus and pubis. These two surgical biopsies came from patients with Pagetic osteosarcoma and samples of the neoplastic tissue were also examined. Five biopsies were taken after the patients had received either calcitonin⁴ or diphosphonate¹ therapy for their Paget's disease but all five still had evidence of active disease.

LIGHT MICROSCOPY

The biopsies were fixed in phosphate-buffered 10% (vol/vol) formalin at pH 7.4 followed by slow decalcification in disodium ethylenediaminetetraacetic acid (EDTA) at pH 6.8 for 10-14 days. They were then processed through to paraffin wax and 5 µm sections cut and stained with haematoxylin and eosin (H & E). Further 5 µm sections were also

Fig. 1 An example of typical Paget's disease of bone (graded 4+) seen in the study. Haematoxylin and eosin $\times 80$

stained with phloxine tartrazine and Mann's and Lepine's methods according to Gurr.⁹ The H & E sections of 25 biopsies exhibited stage 2 disease (lysis and regeneration) on Collin's criteria.⁵ At this point the severity of the disease on representative H & E sections was graded semiquantitatively on a four point scale on the basis of osteoclast and osteoblast numbers, marrow fibrosis, vascularity, residual normal bone or haemopoietic tissue and the presence or absence of a mosaic pattern.

ELECTRON MICROSCOPY

Areas containing osteoclasts, osteoblasts, osteocytes and other marrow cells (including residual haemopoietic foci or fibrotic Pagetic marrow) were marked at light microscopy on the last representative H & E section cut and the corresponding area removed from the block to a depth of 1–2 mm using a sharp razor blade. These were trimmed to a maximum size of 4 $\times$ 6 mm, dewaxed in xylene, rehydrated using decreasing concentrations of alcohol and placed in a cacodylate buffer at pH 7.4 adjusted by sucrose to 300 mmol/kg. The tissue was then postfixed in 1% osmium tetroxide for 1.5 h, passed through increasing concentrations of alcohol (to 100%) and propylene oxide before embedding in Emix resin. Final embedding was in Taab 8 mm flat-bottomed capsules with polymerisation overnight at 60°C. Excess resin was removed from the block, 1 μ m sections cut and stained in 1% toluidine

blue and 1% sodium tetraborate, for the final selection of areas for EM.

Thick sections (80 nm) were cut for EM using a Reichert Ultracut and glass knives. These sections were stained with uranyl acetate in 50% ethanol solution and Reynolds (1963) lead citrate solution.¹⁰ All the sections were viewed in an AEI Corinth 500 transmission electron microscope at an acceleration voltage of 60 kV.

QUANTIFICATION

In 18 of the 25 diseased biopsies the osteocytes, osteoblasts, osteoclasts and other cells present in 1–4 EM grids prepared from representative areas of the paraffin embedded blocks (above) were counted by one observer. The percentage of cells and nuclei containing inclusion bodies was recorded.

Results

ULTRASTRUCTURE OF INTRANUCLEAR INCLUSIONS

Electron microscopy revealed characteristic tubulofilamentous intranuclear inclusions in the osteoclasts of all of the 25 biopsies with histological evidence of Paget's disease. These inclusions consisted of stacked parallel rows or complex whorls of individual filaments often arranged in a compact paracrystalline fashion surrounded by a clear halo of less electron-dense material. The filaments were 12–15 nm in diameter with a central lucent zone 5–7

Fig. 2 Oil immersion light microscopy showing inclusions in four out of 16 nuclei. Toluidine blue. Original magnification $\times 800$

nm in diameter. Clustered filaments seen end on revealed a regular spatial organisation and the inclusions were often associated with electron-dense deposits of fibrillar material. Inclusions could not be seen on the $5\text{ }\mu\text{m}$ H&E, Mann's, Lepine's or phloxine tartrazine-stained sections even using oil immersion. They could, however, be identified by light microscopy in the $1\text{ }\mu\text{m}$ sections stained with toluidine blue prepared for EM (Fig. 2). Low power EM ($\times 1500$ – $\times 3000$) was sufficient to show most inclusions (Fig. 3) but higher magnification revealed the finer detail (Fig. 4) as well as the variability of these structures (vide supra).

INCIDENCE AND SPECIFICITY OF INTRANUCLEAR INCLUSIONS

Inclusions were present in 56–100% (mean = 85%) of the osteoclasts present in each section examined by EM and in 13–64% (mean = 34%) of their individual nuclei (Fig. 5). They occupied from 15–75% of the nuclear cross-sectional area. These structures were not seen in any other type examined (osteoblasts, fibroblasts, endothelial cells, mononuclear precursor and residual haemopoietic cells) and therefore appeared specific to the osteoclast.

INTRACYTOPLASMIC INCLUSIONS

Intracytoplasmic inclusions were seen in 30–40% of the Pagetic biopsies. The dimensions and morphology of these inclusions were similar to the nuclear inclusions but they were not structured in a paracrystalline manner and usually consisted of

haphazard aggregates or stacks of separate filaments (Fig. 6).

HISTOLOGICAL GRADING COMPARED WITH INCIDENCE OF INCLUSIONS

There was a significant relation between the number of affected nuclei in each biopsy and the histological

Fig. 3 Low power EM showing inclusions in two nuclei (arrowed). Seven out of 19 nuclei in the cell showed inclusions. Uranyl acetate and lead citrate $\times 3000$

Fig. 4 A typical paracrystalline inclusion forming a "thumbprint" pattern characteristic of paramyxoviruses. The central lucent zone is seen here. Uranyl acetate and lead citrate. Original magnification $\times 30\,000$

grading of disease severity (Fig. 7). This finding cannot be explained simply by an increased number of osteoclasts per unit sectional area in the more severely affected biopsies as the proportion of affected nuclei was increased. As all 25 biopsies exhibited stage 2 disease, the corollary exercise relating inclusion body load to disease stage could not be performed (but see discussion). There was no correlation between the serum alkaline phosphatase and the incidence of inclusions in the biopsies.

EFFECT OF ANTIPAGETIC THERAPY

Treatment with calcitonin reduced the number of osteoclasts present in bone sections (cf Williams CP *et al*¹¹) but did not affect the morphology or prevalence of the inclusions present. Treatment with the diphosphonate dichloromethylene diphosphonate reduced the number of osteoclasts and caused osteoclast degeneration (cf Alexandre CM *et al*¹²) in the patient examined. Inclusions, however remained identifiable.

ARTEFACTS RESEMBLING INCLUSION BODIES

Discrete intranuclear structures consisting of electron-dense material were also seen in several

cases. On low magnification EM they bear a superficial resemblance to the particular inclusions described earlier (Fig. 8). Higher magnification (Fig. 9) revealed the haphazard appearances of such material, distinctly different from those of the specific inclusions described. These irregular structures may represent some specific, as yet poorly characterised, nuclear abnormality. It is possible however that they may be, more simply, processing or tissue artefacts as similar appearances have been observed not only in other bone disorders but also in a wide range of non-skeletal tissues examined by EM in the same way.

Discussion

The presence of characteristic ultrastructural abnormalities in osteoclasts from Paget's disease was first described by Rebel *et al*⁷ and subsequently confirmed by others.¹³⁻¹⁵ They are unlikely to be pre-necrotic or processing artefacts, or elements of the centriole bodies;^{13,16} nor do they show features and dimensions described for intracellular myofibrils.¹⁷ Goniometric observations have shown them to be distinct tubulofilamentous entities with

Fig. 5 The incidence of inclusions in the osteoclasts of 18 quantified Pagetic biopsies

uniform longitudinal and transverse filaments exhibiting a regular periodicity, irrespective of the overall three-dimensional pattern.¹⁸

We have shown that diseased Pagetic bone biopsies consistently contain these characteristic inclusion bodies. They are confined to osteoclasts. In our study we found a greater proportion of osteoclasts containing inclusions (56–100%) and nine of the 18 biopsies quantified had inclusions in every osteoclast

examined. This contrasts with a lower prevalence reported by Mills and Singer¹³ which may reflect differences either in techniques or patients in the two studies. Gherardi *et al*¹⁵ described two types of cytoplasmic inclusion, which, in their series of eight biopsies were present only in the three patients with the most active disease, as judged by the serum alkaline phosphatase and histological appearance of bone. We observed similar cytoplasmic inclusions in several of our biopsies but these were not confined to the most severely affected cases. We have, however, shown that the prevalence of inclusions within nuclei may be related to the histological severity of the disease (Fig. 7) suggesting that the severity of the disease in any local area of bone may be related to the osteoclastic load of inclusion bodies. The absence of a correlation between the serum alkaline phosphatase and the biopsy inclusion load does not detract from the use of this enzyme as a marker for overall skeletal disease severity, especially as it reflects the overall osteoblastic rather than osteoclastic component of the disease. We have previously studied the specificity of these inclusion bodies for Paget's disease by examining bone biopsies from a wide range of bone disorders.¹⁹ This suggests that the inclusions are highly, though not absolutely, specific for this disease. We and others^{20,24} have noted similar inclusions in the osteoclasts of a few cases of giant cell tumour but with a much lower prevalence, whereas the presence of inclusions in Paget's disease is highly consistent. This has led us to use the presence of these inclusions in large numbers, in conjunction with light microscopy radiographic and clinical features, as an aid in the diagnosis of atypical bone disease.²² Results from our study on the diagnostic potential of the described inclusions (to be published) suggest the absence of a relation between inclusion load and the stage of the disease. This exercise could not be done during the present study, which related inclusion load to disease severity, due to the uniformity of the histological staging in the 25 biopsies; (an association between inclusion load and histological staging is, still however, being investigated).

VIRAL AETIOLOGY OF PAGET'S DISEASE

The presence of these inclusions in all cases of Paget's disease has led to the suggestion that it may be a slow viral disease⁸ analogous to subacute sclerosing panencephalitis (SSPE) and canine distemper. The individual filaments in the inclusions have a marked resemblance to naked measles virus.²³ The inclusion bodies seen on EM are morphologically identical to those present in known paramyxovirus infections *in vitro*²⁴ and *in vivo*.²⁵ The presence of cytoplasmic and nuclear inclusions

Fig. 6 An osteoclast containing an intranuclear and intracytoplasmic inclusion (latter arrowed). Uranyl acetate and lead citrate. Original magnification $\times 5000$

is a common feature of many related viruses, particularly measles.²⁴

Attempts to establish a viral aetiology for Paget's by serological, immunological and tissue culture virus retrieval techniques have met with limited success so far. Also, several centres including ourselves have so far failed to demonstrate a raised complement fixing antiviral antibody for measles in patients with Paget's disease. Only one of 19 patients screened by us had a raised antimeasles titre, a prevalence similar to that in age-matched controls. In contrast, raised antimeasles antibodies are present in patients with SSPE both in serum and in CSF.²⁶ The causative agent of SSPE has also been successfully isolated, examined ultrastructurally and shown to be measles or a closely-related virus.²⁷⁻²⁹ Though Mills *et al*³⁰ have shown in long-term culture of bone explants from Paget's patients multinucleate cells with all the physiological features of osteoclasts, some even containing the typical inclusion bodies already described, attempts to isolate and transfer the virus have been unsuccessful. We are using similar techniques with some variations, to try and demonstrate the infectious nature of these inclusions. Further progress in this particular area will nevertheless be complicated by the absence of a suitable laboratory animal model for Paget's disease. Perhaps virus hybridisation techniques, used for example in the study of herpes simplex and carcinoma of the cervix,³¹ may yield results.

The presence of specific measles antigen have been shown in the Pagetic osteoclasts³² using the immunoperoxidase technique.³³ Together with the morphological appearances of the inclusions this strongly suggests that the inclusions comprise elements of measles paramyxovirus. However, others using immunofluorescence suggest that the particles contain instead, respiratory syncytial virus antigens (RSV).³⁰ It is notable that RSV is another of the paramyxovirus group which shares many morphological similarities.

EPIDEMIOLOGICAL CONSIDERATIONS

Is the proposed viral aetiology compatible with the known epidemiology of Paget's disease?^{1,3-4}

The prevalence of acute measles does not match the geographical distribution of Paget's disease. Thus Paget's disease is very rare in Scandinavia but is common in Lancashire³⁵ whereas the two regions have an equally high incidence of measles. This observation together with the absence in affected patients of specific antiviral antibodies has been held against a viral aetiology.

The absence of antiviral antibodies may be due to methodological problems in recognition, particularly if a viral moiety is involved. It is also possible that a specific infection of the osteoclast itself may preclude an immune response; the osteoclast is an amitotic multinucleate cell formed by the fusion of marrow derived monocytes.³⁶ The predilection of

Fig. 7 Histological grading related to percentage positive nuclei

Paget's disease for certain bones of the skeleton has been explained by a circulating, blood-borne agent and is in keeping with a viral aetiology.³⁷

Inconsistencies between the geographical epidemiology of Paget's disease and measles and those in immunohistochemistry could be explained

Fig. 8 Low power EM demonstrating an intranuclear artefact mimicking a virus like inclusion (arrowed). Uranyl acetate and lead citrate $\times 5000$

by implicating several aetiological factors, one of which is an osteoclastic virus. The virus could be regarded as an acquired endogenous factor (measles or RSV) when other external (exogenous) factors may act, possibly over years or decades, to lead to expression of the disease itself. These exogenous factors might include abnormal concentrations of trace elements in the local water supply, as already considered by Barker *et al* in relation to Paget's disease³⁵ and recently invoked by Triger as a factor in the aetiology of primary biliary cirrhosis.³⁸ The concept of an infectious agent and a second (or several) cofactors in disease pathogenesis is not novel.³⁹

Fig. 9 High magnification demonstrating the structureless nature of the low power inclusion seen in Fig. 8. Uranyl acetate and lead citrate $\times 20\,000$

BONE TUMOURS AND PAGET'S DISEASE

It is interesting to speculate on the aetiological associations of Paget's disease and its neoplastic complications³⁴ of giant cell tumour (osteoclastoma) and osteosarcoma.

Identical paramyxovirus inclusions are inconsistently present, with a lower cellular prevalence in the giant cell tumours of non-Paget patients.^{20,21} Though filamentous inclusions have been reported in some osteosarcomas,⁴⁰ they are different morphologically and morphogenically to those in Paget's disease and giant cell tumours. An increased anti-measles antibody titre absent in giant cell tumours and Paget's disease is noted in osteosarcoma.⁴⁰ There is also epidemiological evidence for an infectious agent in the pathogenesis of the latter.^{41,42} Considering these three diseases it is possible that the timing of the initial exposure to a common infectious agent may determine, after the action of other factors, whether an individual develops adolescent osteosarcoma, giant cell tumour of adulthood or Paget's disease in later life. A model for a similar mechanism has already been proposed for Hodgkin's disease.⁴³ After resection of giant cell tumours do patients have an even higher incidence of Paget's disease compared to the non-tumorous population? Do Paget's patients who develop giant cell tumours or osteosarcoma then develop high titres of specific anti-measles antibody? It will be of interest to note the effect on the frequency of these diseases; firstly of anti-measles inoculation programmes, and secondly following measles epidemics in isolated communities—for example, that recently reported in the Shetlands.⁴⁴

There is therefore some morphological and immunohistochemical evidence for a viral aetiology of Paget's disease and further studies may also shed light on the aetiology of associated neoplasms.

We thank Drs DJ Beard and AJ Patterson for doing most of the bone biopsies, Dr AJ Coup for providing tissue from the two Paget's patients with sarcomatous complications, Dr JR Goepel for the Paget's skull, Dr WH Taylor for a paired set of pre and post calcitonin treated biopsies and Dr JCE Underwood for advice in the preparation of the manuscript. Also we thank Mr M Eaton for photographic assistance.

This research was supported by the Yorkshire Cancer Research Campaign, National Association for the Relief of Paget's disease and the Medical Research and Rehabilitation Trust.

References

- Hamdy RC. Paget's disease of bone, assessment and management. Armour Pharmaceutical Ltd, Praeger Publishers, 1981.
- Collins DH. Paget's disease of bone, incidence and subclinical forms. *Lancet* 1956;ii:51-7.
- Paget Sir J. On a form of chronic inflammation of the bones (osteitis deformans). *Med Chir Trans* 1877;LX:37-63.
- Singer FR, Mills BG. The aetiology of Paget's disease of bone. *Clin Orthop* 1977;127:37-42.
- Collins DH. *The pathology of bone*. London: Butterworths, 1966.
- Rasmussen H, Bordier P. *The physiological and cellular basis of metabolic bone disease*. Baltimore: Williams and Wilkins, 1974.
- Rebel A, Malkani K, Basle M, Bregeon Ch. Particularites ultrastructurales des osteoclastes de la maladie de Paget. *Rev Rhum Mal Osteoartic* 1974;41:767-71.
- Rebel A, Malkani K, Basle M, Bregeon Ch. Is Paget's disease of bone a viral infection? *Calcified Tissue Research* 1977;22, suppl:283-6.
- Gurr TT. *Biological staining methods*. 7th ed. GT Gurr Division, Baird and Tatlock, 1969.
- Reynolds ES. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J Cell Biol* 1963;17:208.
- Williams CP, Meachim G, Taylor WH. Effect of treatment on osteoclast counts in Paget's disease of bone. *J Clin Pathol* 1978;31:1212-7.
- Alexandre CM, Matthews JL, Meunier PJ, Martin JH, Petit H. Ultrastructural aspects of osteoclasts and osteoblasts in Paget's disease of bone treated with dichloromethylene diphosphonate. VIIth International Conference on Calcium Regulating Hormones. Abstract 120, Denver: Sept 5-9, 1980.
- Mills BG, Singer FR. Nuclear inclusions in Paget's disease of bone. *Science* 1976;194:201-2.
- Schulz A, Dellling G, Ruige JD, Ziegler R. Morbus Paget der Knochens. Untersuchungen zur Ultrastruktur der Osteoclasten und ihrer Cytopathogenese. *Virchows Arch [Pathol Anat]* 1977;376:309-28.
- Gherardi G, Lo Cascio V, Bonucci E. Fine structure of nuclei and cytoplasm of osteoclasts in Paget's disease of bone. *Histopathology* 1980;4:63-74.
- Rebel A, Malkani K, Basle M, Bregeon Ch. Osteoclast Ultrastructure in Paget's disease. *Calcified Tissue Research* 1976;20:187-99.
- Ghadially FN. *Diagnostic electron microscopy of tumours*. London: Butterworths, 1980.
- Malkani K, Basle M, Rebel A. Goniometric observations of nuclear inclusions in osteoclasts in Paget's disease of bone. *J Submicr Cytol* 1976;8:229-36.
- Harvey L, Gray T, Beneton MNC, Kanis JA, Russell RGG. Virus-like tubulofilamentous inclusions in the osteoclasts from Paget's disease of bone: a specific phenomenon in osteo-articular pathology? *J Med Microbiol* 1981;14:2PXII.
- Welsh RA, Meyer AT. Nuclear fragmentations and associated fibrils in Giant Cell tumour of bone. *Lab Invest* 1970;22:63-72.
- Le Charpentier Y, Le Charpentier M, Forest M, et al. Inclusions intranucléaires dans une tumeur osseuse à cellules géantes. Mise en évidence au microscope électronique. *Nouv Presse Med* 1977;6:259-62.
- Harvey L, Gray T, Beneton MNC, Douglas DL, Kanis JA, Russell RGG. The diagnostic and aetiological significance of certain ultrastructural features of the osteoclasts in Paget's disease of bone. *Clin Sci* 1981;60:11.
- Madeley CR. *Virus morphology*. Edinburgh: Churchill-Livingstone, 1972.
- Nakai T, Shand FL, Howatson AF. Development of measles virus in vitro. *Virology* 1969;38:50-67.

- ²⁵ Kallman F, Adams JM, Williams RL, Imagawa DT. Fine structure of cellular inclusions in measles virus infections. *J Biophys Biochem Cytol* 1959;**6**:379-92.
- ²⁶ Kiessling WR, Hall WW, Yung LL, Ter Meulen V. Measles virus specific immunoglobulin M response in subacute sclerosing panencephalitis. *Lancet* 1977;**i**:324-7.
- ²⁷ Chen TT, Watanabe I, Zaman W, Mealey J. Subacute sclerosing panencephalitis. Propagation of measles virus from brain biopsy in tissue culture. *Science* 1969;**163**:1193-4.
- ²⁸ Horta-Barbosa L, Fuccillo DL, Sever JL. Subacute sclerosing panencephalitis. Isolation of measles virus from a brain biopsy. *Nature* 1969;**221**:974-5.
- ²⁹ Oyanagi O, Ter Meulen V, Katz M, Koprowski H. Comparison of subacute sclerosing panencephalitis and measles virus: an electron microscope study. *J Virol* 1971;**7**:176-87.
- ³⁰ Mills BG, Singer FR, Weiner LP, Holst PA. Cell cultures from bone affected by Paget's disease. *Arthritis Rheum* 1980;**23**:1115-20.
- ³¹ Elgin RP, Sharp F, McLean AB, McNab JCM, Clements JB, Wilkie NM. Detection of RNA complimentary to herpes simplex virus DNA in human cervical squamous cell neoplasms. (in press).
- ³² Rebel A, Basle M, Pouplard A, Malkani K, Filmon R, Lepatezour A. Bone tissue in Paget's disease of bone. Ultrastructure and Immunocytochemistry. *Arthritis Rheum* 1980;**23**:1104-14.
- ³³ Heyderman E. Immunoperoxidase techniques in histopathology; applications, methods and controls. *J Clin Pathol* 1979;**32**:971-8.
- ³⁴ Singer FR. *Paget's disease of bone*. Plenum Medical Press, 1977.
- ³⁵ Barker DJP, Chamberlain AT, Guyer PB, Gardiner JJ. Paget's disease of bone: the Lancashire focus. *Br Med J* 1980;**280**:1105-7.
- ³⁶ Owen M. The origin of bone cells in the postnatal organism. *Arthritis Rheum* 1980;**23**:1073-80.
- ³⁷ Guyer PB. Radiology in Paget's disease—the skeletal distribution. International Symposium on Diphosphonates in Paget's disease, York 5-7 April 1981 (in press).
- ³⁸ Triger DR. Primary biliary cirrhosis: an epidemiological study. *Br Med J* 1980;**281**:772-5.
- ³⁹ Vianna NJ. Evidence of infectious component of Hodgkin's disease and related considerations. *Cancer Res* 1976;**36**:663-9.
- ⁴⁰ Jenson AB, Spyut HJ, Smith MN, Rapp F. Intracellular branched tubular structures in osteosarcoma. An ultrastructural and serological study. *Cancer* 1971;**27**:1440-8.
- ⁴¹ Morton DL, Malmgren RA. Human osteosarcomas. Immunologic evidence suggesting an associated infectious agent. *Science* 1968;**162**:1279-81.
- ⁴² Harmon TP, Morton KS. Osteogenic sarcoma in four siblings. *J Bone Joint Surg [Br]* 1966;**48B**:493-8.
- ⁴³ Gutensohn N, Cole P. Childhood social environment and Hodgkin's disease. *N Engl J Med* 1981;**304**:135-40.
- ⁴⁴ MacGregor JD, MacDonald J, Ingram EA, McDonnell M, Marshall B. Epidemic measles in Shetland during 1977 and 1978. *Br Med J* 1981;**282**:434-6.

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