labelling.[1] PCNA immunohistochemical expression (evaluated with the PC10 monoclonal antibody) seems to be related to cellular proliferation in many normal tissues and in some neoplasms,[2] such as gastrointestinal lymphomas,[3] central nervous system tumours,[4] lung neuroendocrine neoplasms,[5] and prostatic carcinomas.[6] However, in other tumours, like breast and gastric cancer, PCNA (PC10) expression seems aberrant and not strictly related to proliferative activity.[1 7 8]

Various factors unrelated to cell proliferation may influence the immunohistochemical expression of PCNA, including post-transcriptional regulation (and deregulation) of the PCNA gene,[8 9] long half-life of the PCNA protein,[10] involvement of PCNA protein in DNA repair synthesis,[11] and tissue and section processing—type and ionic strength of the fixatives, fixation time, section heating, immunohistochemical techniques.[8 12 13]

Further problems in PCNA immunohistochemical staining, as in other kinetic quantitative immunohistochemical studies, concern evaluation and scoring methods.[14 15] Should we use quantitative or semiquantitative methods? How many cells should be counted? Which tumour areas should be evaluated (the most positive or random selected areas)? Which immunoreactive cells should be evaluated (all positive cells or only the most intensely stained)?

Particular attention should be also drawn to the kind of antibody used to localise PCNA. Different staining patterns may be seen with different antibodies, and this may add to conflicting and confusing results.[14]

In our opinion PCNA immunostaining should be evaluated with great caution and in some fields even with scepticism. More work is needed to assess the extent and range of PCNA staining in different tissues and lesions (neoplastic and non-neoplastic). PCNA counts should be evaluated concurrently with the different anti-PCNA available antibodies and the results should be compared with other "proliferation markers" and especially with clinical data. The possibility that PCNA immunostaining may have diagnostic[5] or prognostic value[7] is intriguing and carefully performed clinicopathological studies are needed to assess this possibility further. This will be the only way to know if we are faced with an interesting but clinically worthless tool or with an important test to be added to the routine evaluation of neoplasms.

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AgNOR quantification in tumour pathology: What is actually evaluated?

The interest of pathologists in interphase silver stained nucleolar organiser regions (AgNORs) has intensified since it was shown that malignant cells frequently have higher AgNOR numbers compared with corresponding benign or normal cells. Moreover, interphase AgNOR numbers are closely related to cell proliferative activity, suggesting that this parameter might also have prognostic importance.

Nucleolar organiser regions (NORs) are chromosomal segments which contain ribosomal genes. NORs are associated with a group of argyrophilic proteins, and can be visualised by silver staining in routinely processed cytological and histological samples. At light microscopic level AgNORs appear as well defined black dots, which in interphase cells are exclusively distributed throughout the lighter stained nucleoli. Each black dot corresponds, at the ultrastructural level, to a fibrillar centre with the surrounding dense fibrillar component. The number of AgNORs in quiescent cells is generally low (most lymphocytes or stromal cells have only one), while in proliferating cells, such as cancer cells, a high AgNOR number is present.

Over the past six years the silver staining technique has become widespread among pathologists, but the lack of a standardised staining protocol has led to misinterpretation of structures evaluated by different authors.[1] Looking in fact at the micrographs reported—for example, by Giri *et al* (breast carcinoma)[2] Öfner *et al*, (colonic carcinoma)[3] Cheville *et al*, (prostatic carcinoma)[4] and Kaneko *et al* (lung carcinoma),[5] it is evident that not just the AgNORs, but the whole nucleoli have been stained by silver and counted as NORs.

The selective visualisation of AgNORs is subject, apart from the fixative used, to the temperature and temporal length of the staining reaction. These two variables are inversely related to each other: the higher the temperature, the shorter the time required for NOR silver staining. When the staining reaction is prolonged beyond the time for selective visualisation of NORs, all the other nucleolar structures are progressively stained, until the whole nucleolus appears homogeneously stained by silver. It is therefore evident that different nucleolar structures have been stained and counted in various laboratories, and this has caused disagreement about AgNOR numbers reported in individual studies on the same neoplastic lesions.

In a recent investigation it was shown that the total interphase AgNOR area was closely related to the whole nucleolar area stained by silver when staining was prolonged beyond the optimal time for selective interphase NOR visualisation.[6]

To obtain comparable data between different laboratories the whole nucleolus ought to be silver stained and the area occupied by the silver stained nucleoli per cell measured using image analysis instead of AgNOR counting. Because AgNOR area and nucleolar area are so strictly related to each other, the morphometric analysis of silver stained nucleoli will certainly have the same clinical and biological relevance demonstrated for interphase AgNORs.

D TRERÈ
Centro di Patologia Cellulare, Dipartimento di Patologia Sperimentale, Università degli Studi di Bologna, Via San Giacomo 14, 40126 Bologna, Italy.

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Method for grading breast cancer

Parham and colleagues[1] have proposed a new and "simplified" method for grading breast cancer and claim that it is superior to the Bloom and Richardson method,[2] which they rightly criticise for its lack of precision. We agree entirely with this criticism, but are rather surprised that they do not refer to our recent publication in which, for precisely this reason, we have devised modifications which provide objective criteria for the evaluation of the three morphological components of histological grade.[3] We have shown in a study of over 1500 patients that histological grade, using this method, provides powerful prognostic information, and in combination with tumour size and lymph node stage, forms the Nottingham Prognostic Index which can be used by clinicians to stratify patients for

appropriate treatment.[4] This method for histological grade has been adopted by the Royal College of Pathologists' Working Group for use in the NHS Breast Screening Programme.[5]

Parham and colleagues have concluded from a small series of cases (105) that mitotic counts and semiquantitative assessment of tumour necrosis are the most significant factors. Unfortunately, despite their criticism of the Bloom and Richardson method, the authors appear to have fallen foul of exactly the same imprecision which they eschewed. Although they have followed us in defining the field area for mitotic counting, they do not state in their paper how many mitoses per field are allocated for each point scored. Their evaluation of tumour necrosis also lacks clarity. It is admirable to define the dimensions of an area of necrosis but there is surely a flaw in the assessment of *multiple* foci if only the largest focus is counted. On this basis a tumour could have several foci of necrosis each of which might score 1 or 2 points, but this only qualifies it for an overall score of 2, less than a tumour with a single focus scoring 3. The relative lack of numerical data in this paper is also surprising and we are not told the number of cases in each necrosis group. For these reasons we must conclude that not only are there doubts about the reproducibility of this new method but fear that for lack of an adequate description no one else will actually be able to use it.

A number of other points are pertinent. The study is confined to tumours of no special type which seriously reduces its utility, since, as we have shown recently, only 50% of cases of invasive breast carcinoma fall into this category.[6] It is remarkable that no reference is made in this paper to lymph node stage, widely regarded as one of the most powerful prognostic factors available in breast cancer, especially as Fisher and colleagues have shown a close correlation between tumour necrosis and nodal status.[7] Finally, any method which divides patients into four rather than three groups will appear to be more discriminating. We would refer the authors to our paper confirming the utility of the Nottingham Prognostic Index.[5] Using the integer scores five groups of patients are identified with an annual mortality ranging from 1·5 to 32%. In practice, however, prognosis must be related to the available treatment options; in our experience the use of more than three groups serves no useful purpose.

CW ELSTON
IO ELLIS
Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB.

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Dr Parham comments:

Drs Elston and Ellis express surprise that in our paper proposing a simplified method of grading breast cancer[1] we do not cite their recent publication on histological grading.[2] I must confess that while myself and my co-authors may have some favourable attributes, we are not clairvoyants. Our paper was accepted for publication, in its submitted form, on the 1 November 1991 (indicated in the bottom left hand corner of the first page). Their paper was not published until later the same month (8/11/91).

The aim of our study was to produce a simple method of grading breast tumours. The measurement of multiple areas of necrosis, while commendable, would make the method complex and probably less reproducible. For this reason, the largest dimension of necrosis was utilised. For clarity, the scoring of mitotic counts in our paper is the same for both, the new grading method, and the conventional Bloom and Richardson grading method.

Drs Elston and Ellis comment that breast tumours of no special histological type account for only 50% of breast cancers and that this limits the utility of our new grading method. My experience and the findings of others suggest that the figure is nearer 70–75%.[3,4] The remaining tumours, apart from infiltrating lobular carcinomas (accounting for approximately 10% of cases), have special histological features which tend to place them into favourable prognostic groups.

No mention of lymph node stage is made in our preliminary paper, as we concentrated on presenting the prognostic information that can be obtained from the primary tumour. We do, however, state that the combination of the new grading method, with tumour stage and hence lymph node status, may provide further prognostic information. These aspects are currently being investigated.

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Immunophenotype of multinucleated cells in giant cell lesions

I read the interesting paper by Dr Doussis and colleagues[1] and discuss it here in the light of our own results.

In our investigation enzyme histochemistry was applied to cryostat sections of unfixed and undecalcified specimens of 101 different tumours or tumour-like lesions of bone.[2] In all cases the osteoclast-like giant cells showed the same pattern of reactions, which was identical with that of osteoclasts but different from that of the multinucleated neoplastic cells: a lack of demonstrable alkaline phosphatase, but clearly detectable activity of tartrate-resistant acid phophatase (TRACPase) activity; non-specific acid esterase, leucinamino-peptidase, and NADH-tetrazolium oxido-reductase activity. Microdensitometry of the enzyme reaction product[3,4] in giant cells of varying sizes in six different bone tumours exhibited the same trend in all cases: a continuous decline of the relative activities of non-specific esterase and NADH-tetrazolium oxido-reductase, but an increase in the TRACPase activity with increasing cell size. Among the very large giant cells, however, there were cells with both high and very low TRACPase activities. Additional electron microscopic examination showed swollen mitochondria with cristolysis, fragmentation, and swelling of cisternae of endoplasmic reticulum and the nculear envelope, more and larger digestive vacuoles with myelin-like material, and many vacuoles of variable size scattered throughout an electron dense cytoplasm.[2,3] This pattern differed from that seen in the smaller giant cells. Thus we hypothesised that with an increase in cell size osteoclast-like giant cells changed their physiological activities and that at least some of the very large cells degenerated.

It is interesting to note that in the study by Doussis *et al* the pattern of reactivity for anti-CD 68 was quite similar to that of non-specific esterase and NADH-tetrazolium oxido-reductase, because the giant cells with larger diameters clearly showed a lower density of the immunoperoxidase reaction product than the smaller ones (figs 2A and 3A of the paper by Doussis *et al*). We think that these photographs confirm our theory. A microdensitometric examination[4] of these sections would certainly demonstrate a size dependent pattern of the anti-CD68 reaction product comparable with that obtained in the study of the above mentioned two enzymes.

Doussis *et al* show that giant cells of giant cell tumours can be distinguished from other giant cell containing bone tumours by the absence or paucity of the HLA-DR reaction.[1] The authors mention, as one of the possible explanations, that this phenomenon might be due to differences in the nature of the giant cells. But our study of enzyme physiology and ultrastructure of osteoclast-like giant cells in various bone lesions does not support this hypothesis. Furthermore, despite some differences, osteoclast-like giant cells of both giant cell tumours and other giant cell containing tumours or bone lesions share many antigens in common.[1,5–7] Bearing in mind the observation that lymphokines modulate the expression of HLA-DR in human monocytes and macrophages,[8] we suggest that this is also the case for the osteoclast-like giant cells. Therefore, we favour the alternative explanation given by Doussis *et al*, that the differing HLA-DR expression may reflect variations in the tissue matrix or in the immunological response to the neoplasm among the various bone tumours or tumour-like lesions.

K METZE
Department of Pathology, Faculty of Medicine, POB 6111, State University of Campinas, BR 13081 Campinas-SP, Brazil

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