

## Evaluation of nucleolar organiser regions in pulmonary pathology

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Traditional approaches to the pathological diagnosis of neoplasms include macroscopic examination, formalin fixation, and the preparation and examination of paraffin sections stained by various histochemical techniques. These are likely to remain the standard against which alternative and more sophisticated methods are compared. The routine approaches may be disappointing, however, in the assessment of tumours and virtually every day the surgical pathologist is confronted with cases for which light microscopic examination is insufficient to enable a precise pathological diagnosis to be made or prognostic information to be provided. Similarly, the many commercially available antisera for immunohistochemical evaluation may confuse rather than

associated antigen<sup>1</sup>) are among the new techniques that have become available for this purpose. The most recent addition to the list, with possibly one of the simplest applications to current histological practice, is the staining and subsequent study of nucleolar organiser regions.

Abnormalities of the nucleolus have long been known to be a feature of malignant cells.<sup>2,3</sup> Until the advent of electron microscopy, however, nucleolar morphology was of little diagnostic value and consequently of little interest to most histopathologists. The nucleolus has recently been shown to be composed of the relatively electron dense and filamentous nucleolonema and rounded zones of light density and texture known by various names, including *pars amorpha*, *pars fibrosa*, and *fibrillar centre*<sup>4,5</sup> (fig 1). In interphase these structures are equivalent to nucleolar organiser regions, contain the ribosomal genes, and can be visualised at the ultrastructural and light microscopy levels by the remarkably selective staining of their associated proteins by a silver colloid technique.<sup>6</sup> At the light microscopy level nucleolar organiser regions appear as small black dots when stained in this way, known as AgNORs.

Over the past three years numerous pathological and clinical reports have documented new applications of this simple method. The purpose of this review is to give an account of the theoretical and practical basis of the study of nucleolar organiser regions and to document its application to histopathological practice with special reference to the respiratory tract.

### Theoretical and practical basis

Fibrillar centres are the interphase equivalent of nucleolar organiser regions and vary in size according to the degree of nucleolar transcription.<sup>7,8</sup> Their numbers and distribution within the nuclei at the levels of both light and electron microscopy are intimately related to the cell cycle<sup>9</sup> (fig 2). In prophase the dense fibrillar components and granular components of the fibrillar centre disperse and the nucleolus diminishes in size. In metaphase these structures are known to exist on the short arms of the acrocentric chromosomes (13-15, 21, and 22). During this phase they appear as black dots when silver staining is used (fig 3) and as clear areas with the Giemsa banding technique.

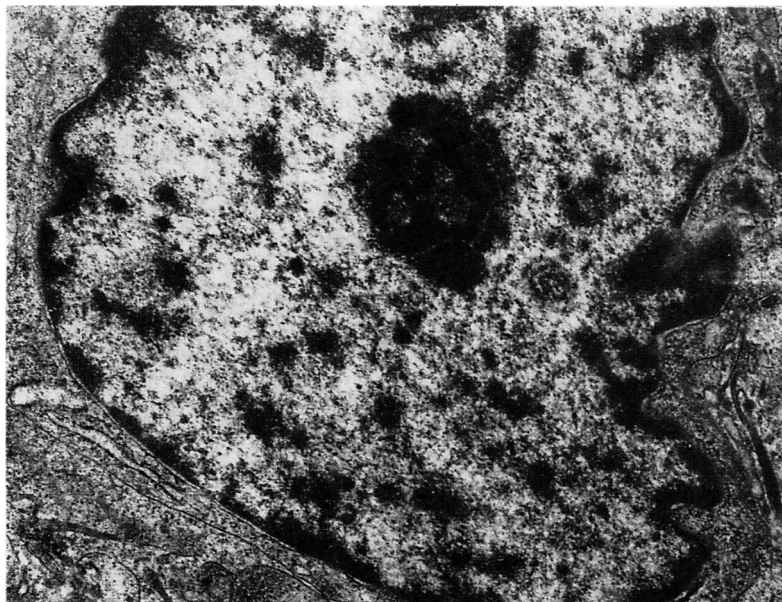


Figure 1 Electron micrograph of a nucleus of a lymphoid cell from a tonsil showing a nucleolus containing three fibrillar centres. From Crocker<sup>33</sup>; reproduced by courtesy of "Current Topics in Pathology."

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assist the inexperienced pathologist. Simple tests that identify malignancy and the degree of differentiation would clearly provide valuable information.

Consequently, there has been growing interest among histopathologists about the role of the nucleus, DNA, and cell kinetics in health and disease. DNA flow cytometry, DNA and RNA "in situ" hybridisation, and the use of the antibody Ki-67 (which detects a proliferation

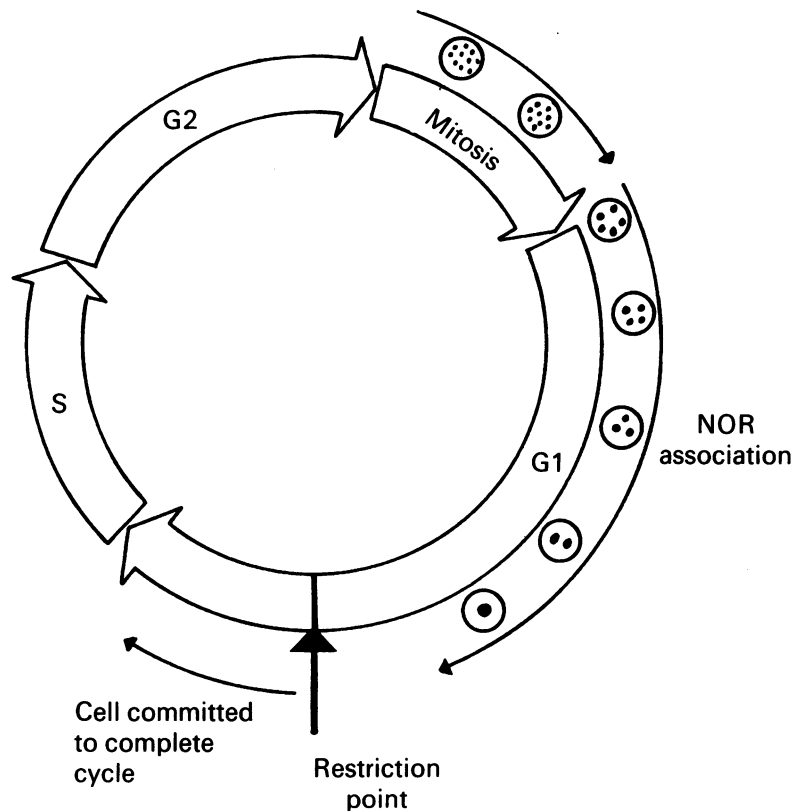


Figure 2 Segregation and aggregation of nucleolar organiser regions (NORs) through the cell cycle. Cells may leave at the restriction point, either permanently or temporarily; the aggregation of nucleolar organiser regions through mitosis up to this point is indicated. G<sub>1</sub> and G<sub>2</sub>—growth phases 1 and 2; S—synthesis phase.

With electron microscopy they appear as areas of relative electron lucency and are known as secondary constrictions.<sup>9</sup> As nucleolar organiser regions occur in constant positions on certain known chromosomes, abnormal siting has been used by cytogeneticists to identify and investigate certain trisomies.<sup>10</sup> In telophase tiny granules associate with the chromosomes

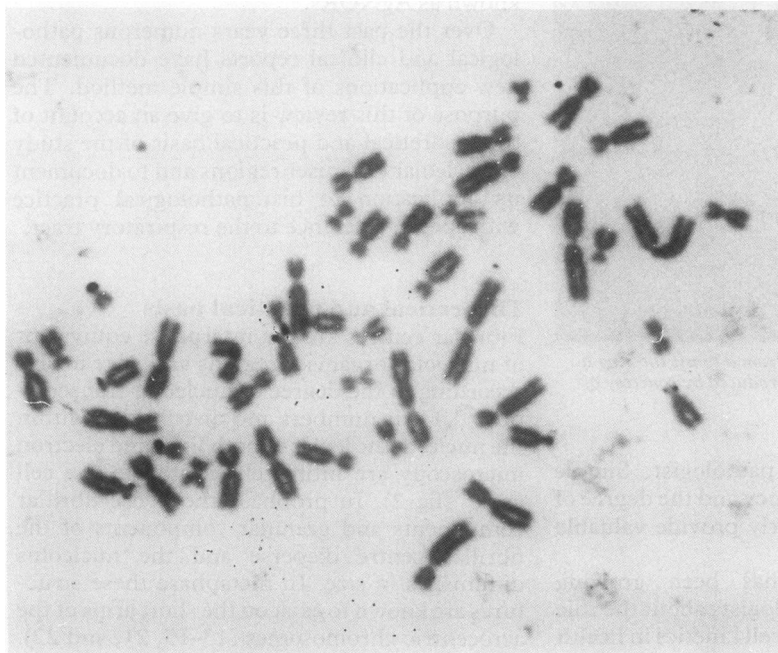


Figure 3 Chromosomes of a normal female human cell (46 XX) stained with the AgNOR method, showing the sites of nucleolar organiser regions on the acrocentric chromosomes. From Crocker<sup>3</sup>; reproduced by courtesy of "Current Topics in Pathology."

bearing nucleolar organiser regions and these granules are arranged further into nucleolar structures. In interphase the nucleolar sub-structure of the nucleolonema and fibrillar centres is re-established.

At a molecular level nucleolar organiser regions consist of segments of ribosomal DNA (rDNA), which are transcribed on to ribosomal RNA (rRNA) and thus ribosomes under the influence of RNA polymerase I. The DNA is in the extended form and the chromatin associated with the nucleolar organiser region has been observed to be dispersed and therefore transcriptionally active.<sup>11</sup> The precise arrangement of chromosomes within interphase nuclei is not understood, but three dimensional reconstruction has shown no numerical relation between the numbers of chromosomes possessing nucleolar organiser regions and the numbers of fibrillar centres.<sup>8</sup> This observation has been confirmed at the ultrastructural level with human oocytes<sup>12</sup> and by light microscopy with certain human neoplastic tissues.<sup>13</sup>

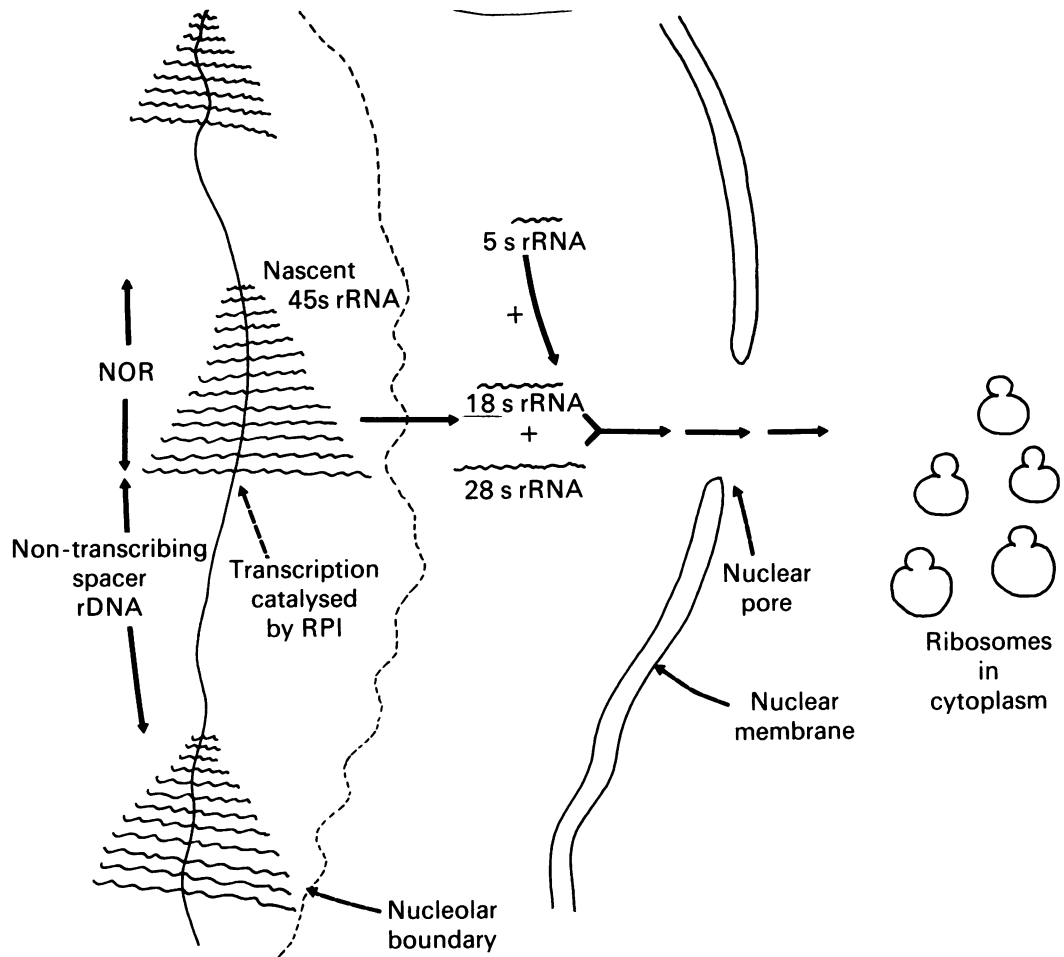
Lysed nuclear or nucleolar spreads on water show nucleolar organiser regions to consist of 20–200 tandem "fir tree" arrays with an rDNA axis and nascent rRNA branches. Attached to these are proteins that process precursor rRNA into ribosome precursors of two sizes (28S and 18S). These are packaged ultimately into the cytoplasm as ribosomes proper (fig 4).

Clearly there have to be regulatory mechanisms and predictably in this context the effects of hormones have been studied. After the incubation of fibroblasts with growth hormone or dexamethasone AgNORs have been shown to be significantly increased in chromosome spreads during metaphase. This suggests ribosome gene activation, and indeed fluorescence studies have shown ribosome gene activation to be closely related to the argyrophilia of nucleolar organiser regions.<sup>18</sup>

In addition to RNA polymerase I, many associated proteins besides RNA polymerase I are associated with nucleolar organiser regions (NORAPs). The most important are C23 protein (nucleolin), B23 protein, and two others (of about 29 and 100 KD). Little is known about phosphoproteins associated with oligodeoxyribonucleotides but they are vital to the study of AgNORs as they are argyrophil by virtue of their carboxyl and sulphhydryl content. These proteins may be specifically identified and localised by various antibodies.

The argyrophil technique appears remarkably specific as a means of detecting nucleolar organiser regions and their associated proteins. Previously, much of the work used hybridisation of radiolabelled rRNA with rDNA, and sequential staining using both methods has shown correspondence between the binding sites.<sup>14</sup> The specificity of the AgNOR reaction has been confirmed further with mercurated fluorescent probes<sup>15</sup> and studies of the localisation of the silver deposition at an ultrastructural level.<sup>16</sup> Fluorescence and immunological methods have also been applied (table), but for general use the argyrophil method has found acceptance largely because of its simplicity.

Figure 4 Schematic representation of the transcription of ribosomal (r) DNA genes ultimately on to ribosomes. NOR—nucleolar organiser region; RPI—RNA polymerase I. From Crocker<sup>33</sup>, reproduced by courtesy of "Current Topics in Pathology."



**Nucleolar organiser regions in histopathological practice**

As the DNA of nucleolar organiser regions is transcribed ultimately into ribosomal RNA and hence leads to protein synthesis, the numbers or sizes of nucleolar organiser regions might be expected to reflect cellular activity, proliferation, or transformation. Many physiological variables affect the numbers of nucleolar organiser regions and their staining intensity. The latter reflects rDNA gene activity, and as actual rRNA synthesis in different subjects measured by uridine uptake can vary up to threefold, individual variation could substantially affect the activity of nucleolar organiser regions, as observed with argyrophil methods. The phases of the cell cycle<sup>9</sup> and certain hormones<sup>18</sup> are known to affect numbers and distribution of nucleolar organiser regions. A reduction of AgNOR numbers with terminal cellular differentiation<sup>19</sup> has been observed in a leukaemia cell line and with aging in stimulated lymphocytes.<sup>20</sup> Despite these

potentially important drawbacks to the application of the argyrophil method to histopathological practice numerous studies have validated the usefulness of AgNOR examination in tumour pathology.

From the early stages it was apparent that uniformity with respect to fixation, technique and enumeration was required and proposals for a standardised approach are outlined below.

**FIXATION**

It has recently been shown that the same distribution of silver stained nucleolar organiser regions (AgNORs) is seen in frozen and paraffin sections, and that sequential staining with various immunohistochemical techniques has no adverse effect on AgNOR number or distribution.<sup>21</sup> It has also been shown that conventional 10% formol saline fixation is perfectly satisfactory, alcohol fixation is optimal, and fixatives containing picric acid and mercury containing fixatives are highly deleterious.<sup>22</sup> The technique is readily applicable to semithin Lowicryl sections<sup>23</sup> and microwave fixed tissue.<sup>24</sup>

**TECHNIQUE**

The reaction was originally devised for chromosome metaphase spreads and runs at room temperature for 60 minutes. The method was subsequently abbreviated from a three step to a one step sequence and modified by lowering the reaction temperature to 20°C. A recent modification has permitted rapid staining (two

*Techniques for identifying nucleolar organiser regions (NORs)*

Reagent	Target
Radiolabelled ribosomal (r)	rDNA
Silver colloid (AgNOR)	NOR associated proteins
Mercuridibromofluorescein	NOR associated proteins
Bismuth ions	100 kD NOR associated protein
Antibodies	Various NOR associated protein epitopes

minutes)<sup>25</sup> but generally a reaction time of 30 minutes gives satisfactory results and is most widely used.<sup>26</sup> Background silver deposition has been related to less than scrupulously clean glassware, residual chloride ions in deionised water, and possibly contaminants in the gelatin.

In brief, the method is as follows. Sections of 3  $\mu\text{m}$  are cut, dewaxed, and taken to water. Deionised distilled water is used to wash and make up the reagents used. The reaction mixture is made by dissolving gelatin (to make a 2 g/dl solution) in 1 g/dl formic acid. This is mixed with 50% aqueous silver nitrate in a proportion of 1:2. Sections are incubated in this mixture for 30 minutes in safelight conditions. The slides are then washed, taken to xylene, and mounted in a synthetic medium. The numbers of stained nucleolar organiser regions in 100 cells of each defined type are then counted and the mean number per cell is calculated.

#### ENUMERATION

Ultrastructural, immunohistochemical, and light microscopic studies have indicated that it is reasonable to suppose that the AgNOR reaction does indeed show nucleolar organiser regions—that is, rRNA genes.<sup>26</sup> Several approaches to their enumeration have been proposed. Firstly, all separate silver stained structures are counted and any adjoining structure, such as a partly disaggregated nucleolus, is treated as a separate feature. Secondly, incubation time is altered so that all subsidiary

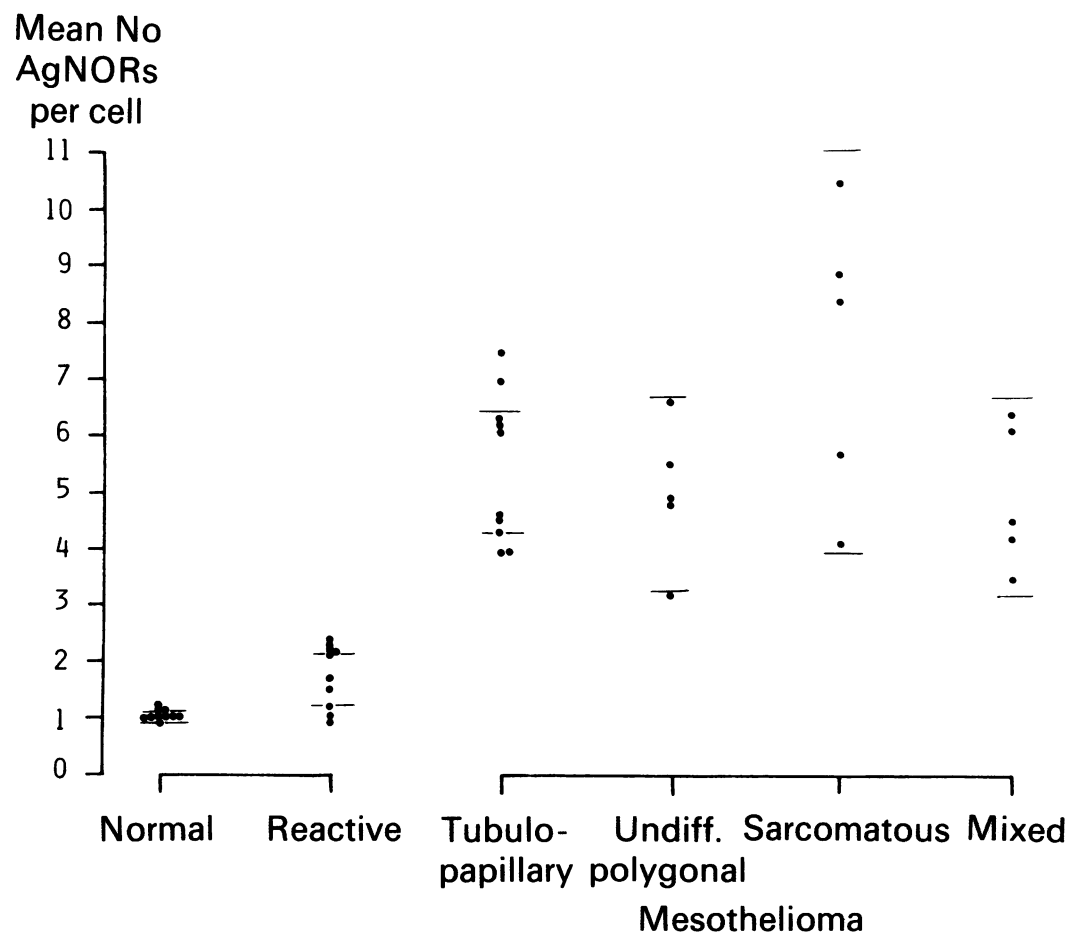
dots within nucleolar associations can be visualised and enumerated. Both methods have been applied to particular diagnostic problems with success and the selection of the enumeration technique should depend on the diagnostic problem. For example, all “dots” must be counted to separate benign from malignant breast tumours, but in the uterine cervix this is not necessary (see below). Neither technique will give absolute counts of nucleolar organiser regions in 3  $\mu\text{m}$  sections of nuclei, which are usually 7  $\mu\text{m}$  or more in diameter. Selective image analysis is not usually satisfactory for counting AgNOR particles but a method of potential use with image analysis is measuring the total silver stained area per nuclear profile.

#### AgNORs and malignant conditions

##### LUNG

Malignant mesothelioma is an uncommon tumour with variable histological appearances. The two major areas of difficulty in diagnosis is differentiation from adenocarcinoma and reactive mesothelial proliferations in histological and cytological preparations. In the past, light and electron microscopy and immunohistochemistry have been applied with some success but these techniques do not distinguish reliably between reactive and neoplastic mesothelial cells.<sup>27</sup> Ayres *et al* showed that simple enumeration of AgNORs reliably separates malignant and benign pleural tissue.<sup>28</sup> Soosay and colleagues<sup>29</sup> found some overlap, however, and

Figure 5 Mean number (with 95% confidence intervals) of stained nucleolar organiser regions (AgNORs) per cell of normal pleura, reactive pleura and the four types of mesothelioma. From Ayres *et al.*<sup>28</sup>



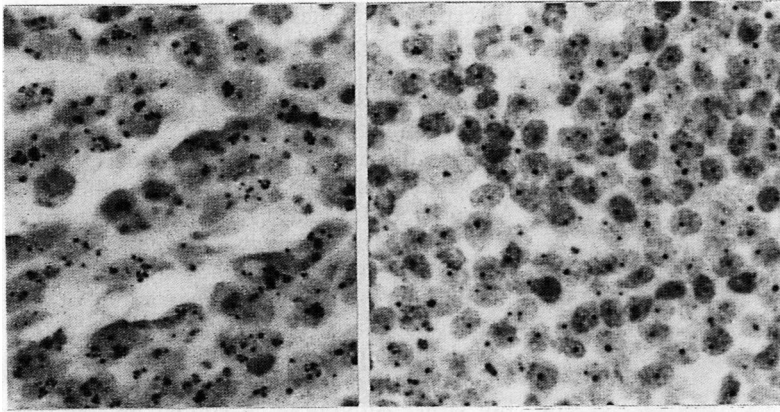


Figure 6 (Left) Bronchial biopsy of oat cell carcinoma stained by the AgNOR method; numerous black dots (NORs) are seen within the nuclei. (Right) Lymphocytes from a bronchial biopsy specimen stained by the AgNOR method with a few black dots within the nuclei.

considered the case for routine use of the AgNOR method in individual problems to be unproved. In (unpublished) work comparing pleural aspirates, pleural biopsy, and follow up postmortem examination we have found the AgNOR method to be a useful adjunct to established methods, though it does not distinguish between adenocarcinoma and mesothelioma (fig 5). A raised AgNOR score can differentiate malignant pleural disease from benign disease.

Small cell carcinoma of the bronchus may also be a diagnostic problem, especially in small biopsy samples obtained at bronchoscopy, where its distinction from other tumours and from aggregates of lymphocytes may be difficult. Enumeration of AgNOR sites has afforded diagnostically useful data in this context. Lymphoid specimens contain less than two AgNORs per cell, whereas small cell carcinoma cells contain over four<sup>30</sup> (fig 6). The values in the two conditions were so widely separated that formal counting was considered unnecessary. The caveat is that low grade non-Hodgkin's lymphoma could not be distinguished from benign lymphocytes and consequently other methods would have to be used to ensure

that the former was not present.<sup>30</sup> None the less, the usefulness of the method in the diagnosis of small cell carcinoma is apparent.

The reproducibility of the method has recently been shown by Benbow and Cromie, who examined small cell carcinoma and typical and atypical carcinoid tumours.<sup>31</sup> The AgNOR counts obtained for small cell carcinoma were similar to those obtained by Crocker *et al.*<sup>30</sup> The values obtained for atypical and typical carcinoid tumours showed considerable overlap with those for small cell carcinoma, in keeping with the concept of a continuous range of clinical behaviour and differentiation.

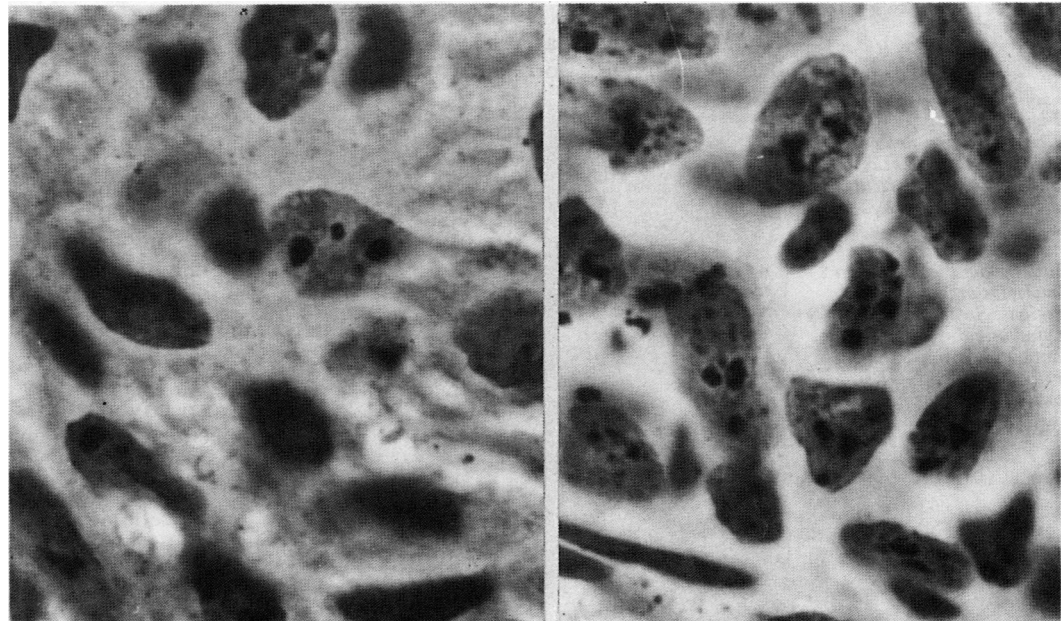
Squamous cell carcinoma of the larynx<sup>32</sup> and bronchus<sup>33</sup> show a general trend towards increased counts with decreased differentiation (fig 7). The technique has been successfully applied to imprints (fig 8) and fine needle aspiration specimens of bronchial carcinoma. Studies of other bronchial carcinomas and the relationship of AgNORs to survival and prognosis are under way.<sup>33</sup> Dual AgNOR and immunohistochemical staining of lung tumours has allowed accurate cellular identification and counting<sup>21</sup> (fig 9).

It is not yet certain whether AgNOR counts can be related to prognosis for the different histological varieties of lung cancer. If, as seems to be the case, AgNOR scores are related to cell proliferation (see below), then such a correlation would be expected. If no relation between prognosis and AgNORs were shown it would imply that factors other than cell proliferation are important for survival in patients with a lung tumour. Factors such as presence or absence of nodal metastases and response to chemotherapy also need to be related to AgNOR scores.

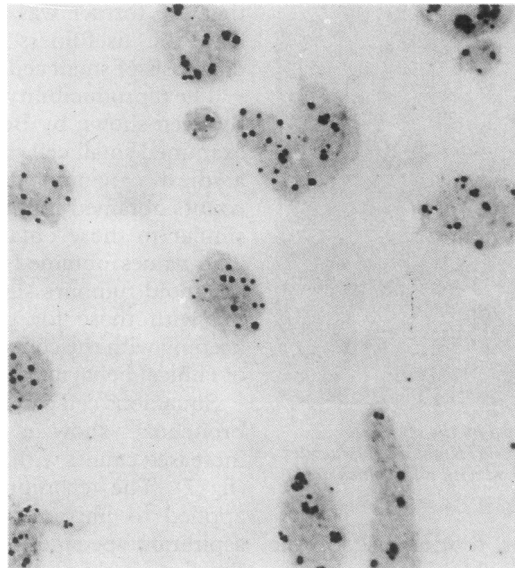
#### OTHER MALIGNANT CONDITIONS

The potential of AgNOR identification and quantification has been investigated in greater detail in malignant diseases outside the lung. The assessment and grading of non-Hodgkin's lymphoma are important because prognosis

Figure 7 (Left) Well differentiated squamous carcinoma of bronchus stained by the silver colloid method for nucleolar organiser regions with a few AgNORs aggregated into nucleolar structures. (Right) Poorly differentiated squamous carcinoma of bronchus stained by the silver colloid method for nucleolar organiser regions, showing more numerous and dispersed AgNORs.



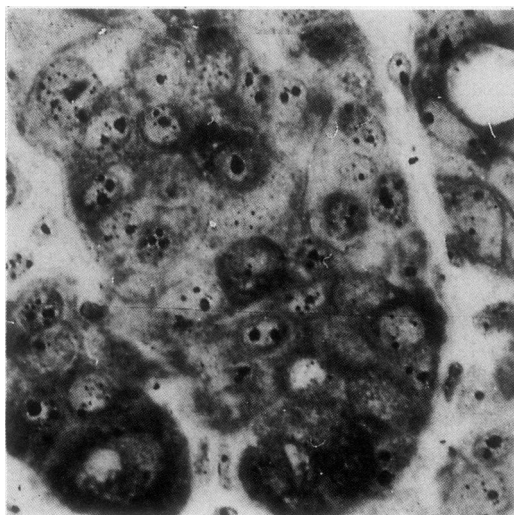
**Figure 8**  
Photomicrograph of a cell imprint of high grade non-Hodgkin's lymphoma stained by the AgNOR method for nucleolar organiser regions, showing numerous AgNORs.



and treatment are helped by accurate evaluation of a battery of special procedures, including light and electron microscopy, immunohistochemistry, histochemistry, and DNA technology. A subjective review of nuclear cytology is an integral part of the classification of non-Hodgkin's lymphoma. Enumeration of NORs in both histological sections<sup>34</sup> and cell imprints<sup>35</sup> also appears to be of value.

It might be expected that the number of AgNORs per nucleus would reflect the number of chromosomes or else ploidy as a normal diploid cell should contain, at most, 10 nucleolar organiser regions (two sets on each of the five acrocentric chromosomes that bear the nucleolar organiser regions). Alternatively, AgNOR numbers might be expected to reflect proliferation and show a numerical relation to either the percentage of dividing cells (S phase cells) or the percentage of cells recognised by the monoclonal antibody Ki 67. This antibody binds to antigen in the nuclei of cells in all phases except when cells which have lost the capacity for division and have temporarily or permanently entered a protracted functional state (Go phase). DNA flow cytometry of non-Hodgkin's lymphoma has shown that the numbers of AgNORs correlate closely with the

**Figure 9**  
Squamous carcinoma of bronchus stained with antibody to low molecular weight cytokeratin by the APAAP method and counterstained by the silver colloid technique for nucleolar organiser regions. Strong cytoplasmic anticytokeratin staining is present with AgNORs visible within the nucleus.



percentage of S phase cells<sup>36</sup> and the percentage of Ki 67 positive cells<sup>37</sup> but not with the ploidy<sup>36</sup> or the number of AgNORs observed in chromosome spreads during metaphase.<sup>13</sup>

The immediate conclusion to be drawn from these studies is that AgNOR numbers in some way reflect or even direct cell proliferation. Morphometric analysis of highly magnified photomicrographs has also proved to be of use in that high and low grade non-Hodgkin's lymphomas show wide separation of the diameter and area of the AgNORs. High grade lymphomas possess many small AgNORs whereas low grade lymphomas possess fewer but larger AgNORs.<sup>38</sup> A similar inverse relation of AgNOR size and number has been observed in cervical intraepithelial neoplasia studied by light microscopy<sup>39</sup> and adenomatous polyps and adenocarcinoma at the ultrastructural level.<sup>40</sup> A similar result is likely to be obtained for lung tumours as clinical and pathological studies progress.

Numerous other tumours and tumour like conditions have been investigated successfully with the argyrophil method. These include salivary neoplasms,<sup>41</sup> certain nasal tumours,<sup>42</sup> cholesteatoma,<sup>43</sup> malignant melanoma and skin tumours,<sup>44-48</sup> breast tumours,<sup>49-53</sup> meningiomas,<sup>54</sup> pituitary adenomas,<sup>55</sup> brain tumours,<sup>33</sup> and various tumours in children.<sup>56-60</sup> In the case of breast disease AgNOR counts have been reported both to be useful<sup>49-50</sup> and to be of limited value<sup>51-52</sup> in differentiating benign ductal proliferation and intraduct carcinomas. This controversy is explained partly by the different techniques used. The technique appears to be less useful in the evaluation of prostatic cancer,<sup>33</sup> thyroid malignancy,<sup>61</sup> colonic mucosal lesions,<sup>62</sup> and gastric epithelial lesions.<sup>63</sup>

#### **Borderline and premalignant conditions** RESPIRATORY TRACT

Dysplastic laryngeal squamous epithelium shows a gradual increase in AgNOR counts as the histological appearance changes from mild to moderate to severe dysplasia.<sup>32-64</sup> This reflects the continuum of numbers seen in dysplastic bronchial epithelium in relation to well, moderately, and poorly differentiated carcinoma of the bronchus.<sup>33</sup> In situ transitional carcinoma of the nose is readily differentiated from its benign and frankly invasive counterparts on the basis of AgNOR counts,<sup>42</sup> suggesting that AgNOR numbers may be useful in indicating the need for closer observation of the patient or further investigation when biopsy specimens are equivocal.

#### **OTHER TISSUES**

Prospective studies of liver biopsies in specimens in which histological appearances could not distinguish between normal liver and cirrhotic livers or between cirrhosis and hepatocellular carcinoma showed AgNOR staining to be of predictive value.<sup>65</sup> Retrospective and comparative studies have shown the method to be of some use in premalignant conditions of the cervix<sup>66-67</sup> and endocervix,<sup>68</sup>

borderline ovarian mucinous tumours,<sup>69</sup> atypical endometrial hyperplasia,<sup>70</sup> and intratubular germ cell neoplasia.<sup>71</sup>

The diagnosis of melanocytic dysplasia is considered by some to be of considerable importance with respect to the future development of malignant melanoma,<sup>72</sup> whereas others challenge the concept of a premalignant melanocytic lesion.<sup>73</sup> Advocates of the former view have failed to show a significant difference in AgNOR counts between clinical and histological melanocytic dysplasia and benign naevi and melanocarcinoma.<sup>74</sup> If an increase in AgNOR numbers heralds malignant change these findings suggest that melanocytic dysplasia is not a premalignant condition.

#### Prognostic use of AgNORs

AgNOR counts have been shown to be closely related to differentiation and inversely related to prognosis in neuroblastomas.<sup>58</sup> They are closely correlated with established prognostic indices in breast cancer<sup>50,52</sup> and may provide a prognostic index independent of the chromosomal number and DNA content of the tumour cells.<sup>51</sup> On the other hand, AgNOR numbers do not predict the biological behaviour of meningioma,<sup>54</sup> rhabdomyosarcoma,<sup>59</sup> Ewing's sarcoma,<sup>60</sup> or thick malignant melanomas.<sup>75</sup> Information on borderline ovarian tumours<sup>69</sup> and dysplastic naevi<sup>44</sup> is incomplete; studies of lung tumours and invasive lesions of cervix are currently under way.

#### Non-neoplastic lesions

An important and extensive use of the AgNOR method may be in the investigation or diagnosis of certain non-neoplastic proliferations and regenerative conditions. For example, the presence of more than four AgNORs may be used to differentiate cholesteatoma from normal squamous epithelium.<sup>43</sup> The technique is of use in the differentiation of xanthogranulomatous pyelonephritis and clear cell carcinoma.<sup>76</sup> A physiological increase of AgNORs is observed in pituitary corticotrophs after adrenalectomy in adult male Sprague-Dawley rats.<sup>77</sup> AgNORs are a sensitive indicator of tubular damage and degree of recovery and will provide a useful aid to clinical management of patients with early dysfunction of renal allografts.<sup>78</sup>

#### Conclusions

Over the past three years numerous publications have helped to show the usefulness of AgNOR enumeration. The argyrophil technique is remarkably specific for proteins associated with nucleolar organiser regions and a simple and a standardised approach has allowed correlation of data within and between laboratories. Relatively few prognostic studies have been carried out, and benign and malignant conditions in some studies have a considerable overlap of AgNOR numbers.<sup>32,54,55,61</sup> The method at present cannot distinguish between physiological and neoplastic proliferation in certain lesions—a failure shared with other innovative techniques in tumour pathology, notably immunolabelling with Ki 67 and

DNA flow cytometry. Nevertheless, the initial promise of the method has been fulfilled in many areas. The technique has found use, for example, in neoplasms of skin,<sup>44,46</sup> breast,<sup>49</sup> lymphoid tissue,<sup>34-36</sup> kidney,<sup>64</sup> and liver<sup>65</sup> and in childhood cancers<sup>56-58</sup> and mesothelial tumours.<sup>28</sup> Interesting and potentially useful information has been provided for many types of neoplasms, including certain canine tumours.<sup>79</sup> These suggest that nucleolar organiser regions reflect or even direct cell proliferation, and the numbers may reflect complex cellular and nuclear interactions, including gene amplification.

Future studies using antibodies to nucleolar organiser regions and their associated proteins or other probes should elucidate their role in the transformation of tumour cells, and explain the ectopic situation of some AgNORs outside nucleolar structures. It is hoped that such studies will also provide further diagnostic applications.

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