Correlation between PCNA and AgNOR scores in non-Hodgkin's lymphomas using sequential staining technique

F G Smith, P G Murray, J Crocker

Abstract

Aims: To investigate the relation between the numbers of interphase silver stained nucleolar organiser regions (AgNORs) and immunolabelling with the monoclonal antibody PC10, which demonstrates proliferating nuclei by reacting with proliferating cell nuclear antigen (PCNA).

Methods: The established sequential technique for the demonstration of interphase AgNORs and of antigens in paraffin wax sections was applied to a small series of non-Hodgkin's lymphomas (NHL) of both low and high grade type, together with specimens of palatine tonsil. The numbers of PCNA positive cells were counted in all specimens; in the tonsils the cells were counted in both follicle centres and in the interfollicular areas. The numbers of AgNORs in both PCNA positive and PCNA negative nuclei were then counted.

Results: Lower numbers of PCNA positive cells were found in the low grade than the high grade NHL (18.28.4% and 34.2-51.3%, respectively). This was reflected in the two areas of the palatine tonsils, the counts being higher in the follicle centre nuclei than in those in the interfollicular compartments. In general, the numbers of AgNORs were higher in the PCNA positive nuclei than in those lacking the antigen; however, a consistent finding was that relatively high AgNOR scores were observed in PCNA negative nuclei, especially in the high grade lymphomas. In the tonsils, however, the AgNOR counts were much lower in the nuclei lacking PCNA than in those containing it.

Conclusions: The results obtained in the lymphomas were rather unexpected as, in general, previous studies have shown a close direct or indirect relation between AgNOR scores and proliferating cell counts. An explanation for these findings may be that the argyrophil proteins associated with proliferating cells remain in the nucleus in detectable form longer than the PCNA antigen, at least in neoplastic tissue.

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The scoring of interphase NOR numbers using the argyrophil (AgNOR) technique¹ has been

shown to be of use in assessing the degree of malignancy in neoplasms.²⁻⁴ In the case of non-Hodgkin's lymphomas (NHL), AgNOR numbers can help to distinguish between high and low grade lymphomas^{5,6} with greater scores in the high grade than in the low grade types. However, the reason for the increased count in the high grade lymphomas was not originally fully understood. In view of the constant chromosomal positions of NORs, it might be expected to reflect cell ploidy, but there is now a great volume of evidence showing that interphase AgNOR scores represent the level of cell proliferation.

NORs are ribosomal DNA cistrons found in the short arms of the five human acrocentric chromosomes (13, 14, 15, 21 and 22).²⁻⁴ During interphase the NORs are represented by nucleolar fibrillar centres which appear as black "dots" of varying size when stained by the AgNOR method. This technique makes use of the fact that NOR associated proteins bind silver.

The double staining method using monoclonal antibodies and the argyrophil technique¹ has helped to overcome the problem of the heterogeneity of neoplasms by allowing the identification of cells before AgNOR enumeration. We applied this method to NHL using the PCNA antibody PC10. Unlike the Ki67 antibody which has previously been used in the double staining of NHL,⁷ this PCNA antibody reacts with paraffin wax sections and is not limited to frozen sections.

Methods

Normal palatine tonsils and a series of four low grade and six high grade NHL were selected from the files at East Birmingham Hospital. These had been fixed for between 24-48 hours in 10% formol-saline and taken to paraffin wax in the usual way. Sections were cut at 3 µm thickness, allowed to adhere to glass slides at room temperature, and then incubated at 37°C overnight. Sections were then dewaxed and taken to water.

Sections were incubated in mouse monoclonal anti-PCNA antibody (Dako PC10) at a dilution of 1 in 100 in TRIS-buffered saline (TBS) (pH 7.6) and then washed in TBS. PCNA was visualised using the standard alkaline phosphatase-anti-alkaline phosphatase (APAAP) labelling method.⁸ Adjacent sections were also stained for T cell activity by means of a polytypic antibody to CD3 (Dako) and B cell content with a monoclonal antibody to CD20 (Dako L26).

Department of
Histopathology, East
Birmingham Hospital,
Bordesley Green East,
Birmingham B9 5ST
F G Smith
J Crocker

The Biomedical
Research
Laboratories,
Wolverhampton
University
P G Murray

Correspondence to:
Dr J Crocker
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After the sections were washed in distilled deionised water for 10 minutes, standard AgNOR staining was performed.^{5 9 10} Incubation at room temperature in AgNOR staining colloid was extended to two hours to improve contrast. Sections were washed twice in distilled water for 10 minutes each and mounted in aqueous mounting medium.

Sections were examined under a $\times 100$ oil immersion objective lens. AgNORs were counted in 200 PCNA positive and PCNA negative cells in each case, this number having been reached by the standard continuous mean technique. In each of the tonsils, counts were performed separately for the germinal centres and interfollicular areas. Nuclei were taken as being positive only if there was an unequivocal, intense red reaction product. The differences between PCNA scores in the different groups of specimens and between AgNOR counts for different cell groups were analysed by means of confidence interval analysis.

Results

A positive reaction to the PCNA antibody resulted in an intense red colouration of the nucleus. The AgNORs showed up as black

"dots" of variable size. (figs 1 and 2). The lymphomas were all of B cell phenotype as shown by CD 20 staining and where there was an intervening "reactive" T lymphocytic infiltration these (CD3-positive) cells had only 1.0 to 1.5 AgNOR dots per nucleus and accounted for no more than 5% of the total cell count.

In the high grade lymphomas the PCNA positive cells ranged from 34.2 to 51.3%. These positive cells had mean AgNOR scores from 5.66 to 9.93, while the PCNA negative cells had a mean AgNOR score from 2.76 to 6.62 (table).

In the low grade lymphomas PCNA positive cells ranged from 18.0 to 28.4%. The positive cells had mean AgNOR scores from 5.4 to 8.39, while the PCNA negative cells had a mean AgNOR score from 1.49 to 4.63 (table).

The PCNA score in tonsillar follicle centres ranged from 43.0 to 50.0% while in the interfollicular areas it was 2.0 to 7.5%. In follicle centre cells the AgNOR scores for the PCNA positive nuclei were from 1.9 to 6.89 and in interfollicular cells the range was from 1.67 to 6.79. The cells lacking PCNA activity in follicle centres had a mean AgNOR score of 2.09 to 3.05; in the interfollicular PCNA negative cells the AgNOR score was from 1.45 to 1.67 (table).

Although the numbers of specimens were small, the quantity of data permitted statistical evaluation. At a 95% confidence interval, the difference between the AgNOR scores in PCNA positive and negative cells in high grade lymphomas was significant at $p < 0.05$. In low grade lymphomas, the difference between the AgNOR scores in PCNA positive and negative specimens was significant at $p < 0.01$. In interfollicular and follicle centre cells of the tonsil there was a significant difference in AgNOR counts between PCNA positive and negative cells at $p < 0.01$ for both types of cells.

Discussion

The counting of AgNORs is becoming increasingly used in tumour histology for assessing the degree of malignancy and consequently, in some cases, also for the prognosis and treatment of the patient. Many methods, including electron microscopy, enzyme histochemistry, immunochemistry, morphometry and DNA flow cytometry have been used to help with the grading of NHL, and within recent years NOR enumeration has also been of value in their assessment. The high grade lymphomas have higher AgNOR scores than the low grade specimens.^{5 6} So far the reason for this observation is not completely understood. It was originally presumed that high AgNOR scores could represent cell ploidy, which is known often to be excessive in NHL. To test this idea DNA flow cytometry was performed on both high and low grade lymphomas,¹¹ comparing the mean AgNOR count with the DNA index and the percentage of S phase cells (proliferating cell numbers). There was no correlation between the AgNOR score and DNA aneuploidy ($r = 0.14$), but the AgNOR score

Figure 1 Tonsillar follicle centre, showing mainly PCNA positive (red) nuclei, most of which contain multiple black dot-like "AgNORs".

Figure 2 Centroblastic lymphoma, showing large PCNA positive (red) nuclei, containing multiple "AgNORs". Some PCNA negative nuclei also have several AgNOR sites.

Mean AgNOR scores in PCNA positive and PCNA negative cells

Specimen	% PCNA positive cells	Mean AgNOR score in PCNA positive cells (Range) (SEM)	Mean AgNOR score in PCNA negative cells (Range) (SEM)
Centroblastic	39.7	9.93 (4-15) (0.27)	6.62 (1-9) (0.21)
Centroblastic	44.7	5.66 (3-10) (0.16)	2.78 (1-7) (0.14)
Immunoblastic	51.3	9.06 (5-14) (0.18)	5.32 (1-9) (0.20)
Immunoblastic	37.9	8.59 (4-18) (0.26)	4.12 (1-9) (0.18)
Immunoblastic	34.2	8.93 (4-22) (0.41)	5.30 (1-10) (0.19)
Lymphoblastic	41.5	9.24 (5-16) (0.27)	4.63 (1-9) (0.21)
Lymphocytic	28.4	5.40 (3-10) (0.23)	1.49 (1-5) (0.07)
Centrocytic	18.0	7.71 (4-11) (0.29)	2.78 (1-9) (0.17)
Centroblastic/centrocytic	19.3	7.71 (5-12) (0.25)	4.63 (1-8) (0.16)
Centroblastic/centrocytic	26.3	8.39 (5-14) (0.32)	3.11 (1-9) (0.18)
Tonsil FC	46.5	5.16 (2-9) (0.16)	2.09 (1-6) (0.10)
IF	2.0	3.75 (3-5) (0.48)	1.55 (1-5) (0.06)
Tonsil FC	50.0	1.90 (2-10) (0.19)	2.34 (1-6) (0.12)
IF	7.5	1.67 (2-8) (0.45)	1.45 (1-4) (0.05)
Tonsil FC	43.0	6.80 (3-11) (0.23)	2.96 (1-7) (0.14)
IF	3.0	3.13 (2-12) (1.40)	1.67 (1-6) (0.07)
Tonsil FC	45.0	6.89 (2-11) (0.21)	3.05 (1-7) (0.14)
IF	7.0	6.79 (4-10) (0.48)	1.54 (1-4) (0.06)

FC = follicle centre; IF = interfollicular area.

correlated strongly with the percentage of S phase cells ($r = 0.86$). Therefore it seemed that AgNOR scores somehow reflected the level of cell proliferation. More direct experiments were carried out using karyotypic analysis and again it was shown that AgNOR numbers were related to cell proliferation rather than chromosomal ploidy.¹²

To test this, further experiments using the antibody Ki67¹³⁻¹⁵ were performed. Ki67 is an antibody believed to bind to the nuclear membrane of proliferating cells. However, a problem with Ki67 is that frozen sections are essential as the epitope with which it reacts is destroyed when paraffin wax sections are produced. Initial experiments¹⁶ compared Ki67 scores in frozen sections with AgNOR scores in paraffin wax sections from the same cases for a large series of NHL. There was a good correlation between the two sets of data ($r = 0.86$). This study was expanded using the double staining technique¹ for sequential immunostaining and NOR silver binding. Ki67 labelling and AgNOR counts were thus obtained from the same section in each of the cases. The results showed that the Ki67 positive cells had high AgNOR counts while the Ki67 negative cells had low AgNOR counts, in both high and low grade lymphomas.⁷ This provided further evidence for a correlation between AgNOR scores and cell proliferation.

Until recently there was little information with respect to this relation. It had been shown that counts increased in stimulated lymphocytes¹⁷ and decreased in differentiating melanoma¹⁸ and HL60 cell lines.¹⁹ However, the picture is complicated by a recent investigation using the U937 promonocytic cell line.²⁰ It was shown that the level of differentiation in the U937 cell line, when induced by a variety of cytokines, was one contributory factor to the decreasing AgNOR count. The doubling time was constant during each experiment and thus in this instance the rate of proliferation was apparently of lesser importance. The fall in AgNORs may have been due to their fusion which is known to occur during the cell cycle as cells exit from mitosis. None the less, it has been shown in a number of neuroblastoma cell

lines that cell proliferation alone is related to AgNOR numbers.²¹⁻²² However, in these experiments the total amount of argyrophilia was calculated and not the number of AgNORs; as not all argyrophilia is due to transcriptionally active molecules the amount may not necessarily change in proportion to the cells' proliferative state. It seems that some of the AgNOR associated proteins to which the silver binds may have structural roles.

In the current study we have further investigated the relation between AgNOR scores and cell proliferation using the PCNA antibody which unlike Ki67 reacts with paraffin wax sections. We found that the proliferating PCNA positive cells had higher AgNOR scores than the PCNA negative cells, and that both the numbers of PCNA positive cells and AgNORs were greater in high grade lymphomas than low grade lymphomas. However, the AgNOR counts were relatively high in PCNA negative cells (1.5-6.6) compared with counts in non-proliferating cells as shown by the interfollicular counts in the tonsil sections (1.5-1.7). The PCNA negative cell scores of the NHL specimens were also greater than the PCNA negative scores in the tonsil germinal centres (2.1-3.0). This may indicate that PCNA and AgNORs are presented at different stages of the cell cycle or may linger for different durations after mitosis. It is of interest that the cells lacking PCNA in both low and high grade lymphomas may still have relatively increased AgNOR scores compared to resting lymphoid cells. This may reflect a lack of differentiation rather than entry into cell cycle.²⁰ The small numbers of reactive T cells present in the specimens would appear to have low AgNOR scores. Thus although we have shown an association between PCNA labelling and AgNOR counts in NHL and therefore, yet again, an association between cell proliferation and AgNOR scores, it appears that PCNA negative cells still have relatively high counts in lymphomas. An identical phenomenon has recently been demonstrated in hyperproliferation of epithelial cells of the rat stomach.²³ This may result from the production of NORs lagging behind the PCNA epitope production; consequently this requires further investigation. It remains a mystery, however, to account for the apparently close relation which has been shown on several occasions between AgNOR and Ki67 counts for a variety of tumours. We still have much to pursue in relation to our understanding of the interrelations between the increasingly large numbers of proposed "markers" of cell proliferation.

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