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# Evaluation of AgNOR expression in human biopsy samples of lymphoid tissue.

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***SUMMARY***

*We hypothesize that separate analysis of AgNOR contents in cells previously identified as type I or type II cells (cells with low and high content of AgNORs respectively) miht help to define the relationship, if any, between changes in the expression of AgNORs and their malignancy or variations in cell cycle parameters. By using this method. the final measurement would be free of the bias introduced by the different proportions of type I an Type II cells within the sample which, as previously demonstrated, is related to the growth fraction.*

*Human biopsy samples corresponding to reactive lymph nodes (RLN), Hodgkin's disease (HD) and non Hodgkin's lymphomas (NHL) were used for this study. Cells with a high and low content of AgNORs (type I and type II cells respectively) were directly identified by the observer and the mean area of AgNORs of each type of cell was separately calculated by means of image processing.*

*The mean AgNOR area of type I cells was almost superimposable in the three study groups. As regards type II cells, no difference was found between NHL and RLN, but in HD the mean AgNOR area was greater than in any other group. In RLN and NHL, the mean area of AgNORs of type II cells was six times as high as that of type I cells, while in HD thisproportion was greater.*

*These results further support the hypothesis that changes in the expression of AgNORs, as assessed in tissue sections, are mostly linked to cell proliferation, such changes mainly being due to the variation of the relative numbers of resting (type I cells) and proliferating (type II cells) rather than to modifications of the mean AgNOR content of individual cells.*

*Key words:* AgNORs. Quantification. Lymphoid tissue. Human biopsy. Proliferation.

## INTRODUCCION

A precise relationship between modifications in the expresion of argyrophihilic nucleolar organizer regions(AgNORs) and the physiological events which they express is an arduous task that has not yet been completed. A number of reports has been made in which a close relationship between increased expression of AgNORs and increased proliferative activity is suggested (1). Experimental work done in our laboratories shows that in rat thymocytes the expression of AgNORs increases through the cell cycle (2) and that an approximation to the growth fraction can be obtained both in rat thymic sections (3) and in human biopsy samples of lymphoid tissue (4) by calculating the proportion of cells with different AgNOR contents (type I - and type II - cells, cells with low or high content of AgNORs, repectively).

Mean AgNOR content per cell has also been reported to correlate with malignancy (5) and with cell cycle parameters such as the cell doubling time (6). Most often, when the mean area and/or numbers of AgNORs per cell are used to evaluate modifications in the

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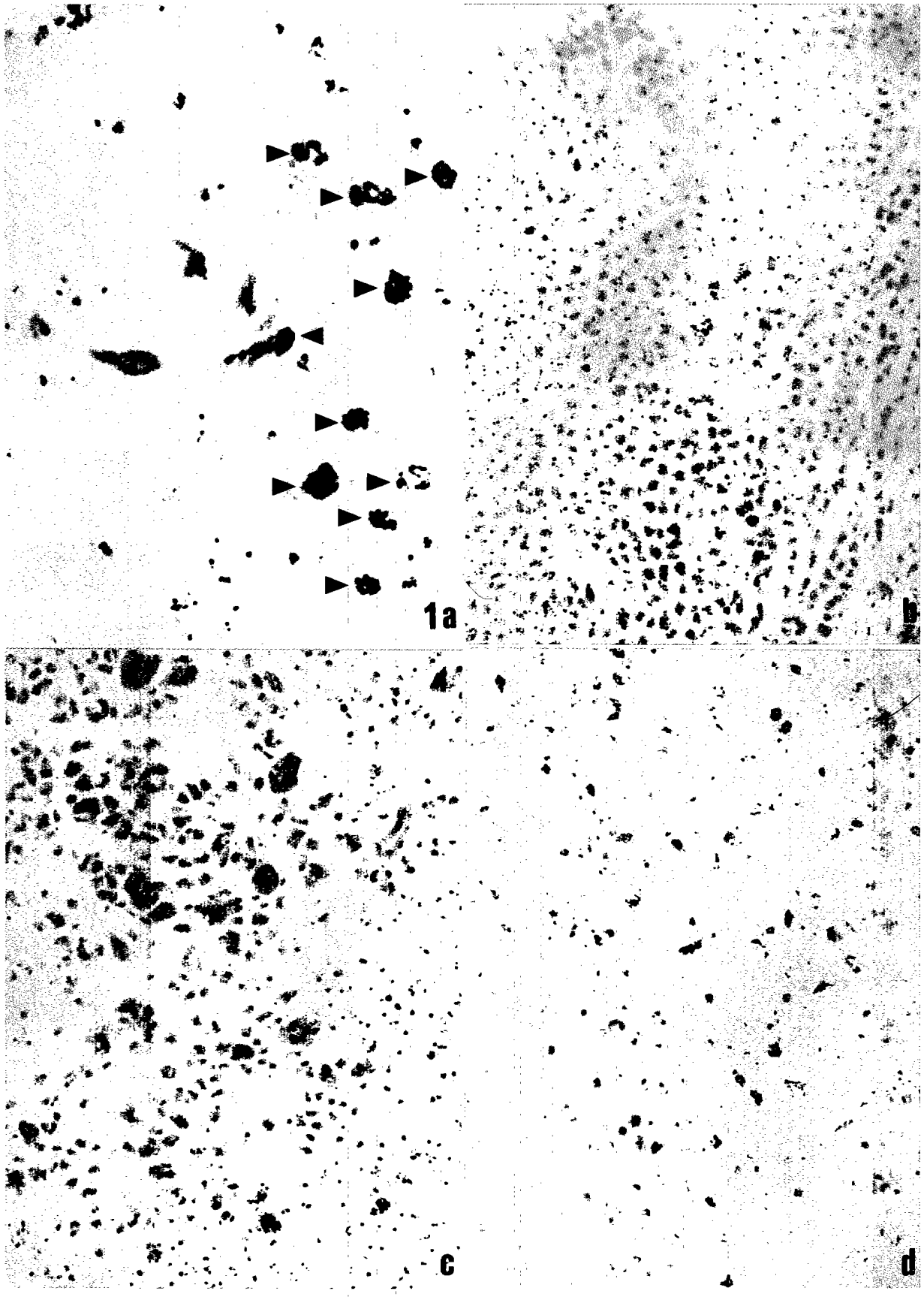


Figure 1.- Silver-stained nucleolar organizer regions (AgNORs) in human biopsy samples of lymphoid tissue. a) Section from a high-grade lymphoma in which cells with a high expression of AgNORs (type II cells: arrowheads) are easily distinguishable. The proportion of type II cells tended to be low in RLN; (b) intermediate in HD; (c) and high in high grade HL (d). a: X 280; b-d: X 150.

expression of AgNORs, a sample of a certain number of cells (50-100) is taken at random. By doing so, the final measurement depends not only on the quantity of AgNORs per cell but is also related to the number of cells that, within the sample, have an increased quantity of AgNORs.

We hypothesize that separate analysis of AgNOR contents in cells previously identified as type I or type II cells might help to define the relationship, if any, between changes in the expression of AgNORs and either malignancy or variations in cell cycle parameters. By using this method, the final measurement would be free of the bias introduced by the different proportions of type I and type II cells within the sample which, as previously mentioned, is related to the growth fraction (4). To check this hypothesis, the AgNOR contents of type I and type II cells, as directly identified under the microscope, were quantified and compared in benign and malignant samples of lymphoid tissue. We then tried to gain insight into the relationship between modifications in the expression of AgNORs and either proliferative rate or malignancy in samples of lymphoid tissue.

## MATERIALS AND METHODS

### Samples.

Biopsy specimens (n=50) of lymphoid tissue were studied. The series included 18 reactive lymph nodes (RLN), 12 Hodgkin's disease (HD) and 20 non-Hodgkin's lymphomas (NHL) classified according to the criteria of the Working Formulation into low-grade (n=7), intermediate-grade (n=4) and high-grade lymphomas (n=9).

Representative samples were taken from each case. At least 3 frozen sections (3-5 μm thick) from each specimen were incubated (15 minutes, 37°C, in the dark in moist chamber) with silver colloid prepared as previously described (7). Then sections were thoroughly washed in water, lightly counterstained with methyl green, dehydrated and mounted with DPX.

### Evaluation of AgNORs.

AgNORs were studied with a MIP image analyzer (Microm España, Spain). An aberration-free 50 X objective lens (Carl Zeiss Jena, Germany) was used. The image projected onto the CCD used for image capture was mapped into a 512x512x24 bit memory. The content of the frame memory was equivalent to 7498 $\mu m^2$. Ten microscope fields were randomly selected among the areas of interest on each slide. For morphometric purposes, cells were classified as type I or type II according to the expression of AgNORs (3,4). AgNOR contents were evaluated in 125 type I and type II cells in each sample and expressed as the mean area of AgNORs per cell (2). The ratio between the mean area of AgNORs of type II and type I cells was calculated for each case.

### Statistics.

Comparison of AgNOR-derived parameters after morphometry was performed by ANOVA at a level of significance of 95%. Scheffe F-test was used for two group comparisons.

## RESULTS

Silver staining resulted in clearly identifiable intranuclear black dots in all the specimens of our series (fig.1). Some cells showed one or two small AgNOR dots in the nucleus (type I cells) while others (type II cells) were characterized by larger AgNOR dots which varied in number and frequently appeared as clusters of silver-stained dots (fig. 1a). The proportion of both types of cells varied considerably among different samples, regardles of histological grade or type. Despite this, the proportion of type II cells tended to be low in RLN and low grade NHL, intermediate in HD and high in high grade NHL (fig. 1, b-d).

Numerical data of the morphometric study are shown in table I. Irrespectively of the study group (RLN, HD and NHL) the mean area of AgNORs of type II cells was greater ($P<0.05$) than that of type I cells. In RLN and NHL the mean area of AgNORs of type II cells was around six times as high as that of type I cells, while in HD this proportion was greater ($P<0.05$). The mean area of AgNORs of type I cells was almost superimposable in all three study groups. As regards type II cells, no difference was found between NHL and RLN but in NHL the mean area of AgNORs of type II cells was greater ($P<0.05$) than in type II cells from any other group.

## DISCUSSION

In the last decade, AgNORs have received the attention of researchers and pathologists since they have been considered as an "open window" to certain intimate processes of cell physiology such as cell proliferation or cell differentiation and maturation. A number of publications have appeared on the histo- and cytopathological application of the evaluation of AgNOR particles identifiable within the nucleus of inerphasic cells. The chief purpose of these studies has undoubtlees been to determine whether the evaluation of AgNORs can be used to discriminate between benign and malignant entities and whether the evaluation of AgNORs may be of any help in defining the prognosis of malignant neoplasms (1).

There is a growing body of evidence supporting the notion that the quantity of AgNORs is related to cell proliferation (2,6,9,10). A number of reports have also been made on attempts to establish a correlation between the expresion of AgNORs and malignancy. In this sense, an increased expression of AgNORs has been described

**Table I.** Mean area of AgNORs per cell ($\mu m^2$)

| | | | |
|---|---|---|---|
| Ratio type II/type I cells | | | |
| RLN | 1.0±0.1 | 6.7±0.7 | 6.3±0.6 |
| NHL | 0.9±0.1 | 5.8±0.5 | 6.5±0.5 |
| HD | 1.0±0.1 | 8.5±0.9* | 8.4±0.9* |

Mean area of AgNORs per cell as calculated in type I and type II cells from reactive lymph nodes (RLN), non-Hodgkin's lymphomas and Hodgkin's disease (HD). The ratio between mean AgNOR area in both types of cells is also shown for each group. Values are mean ± SEM. * Statistically different from data of the same column ($P<0.05$ Scheffe F test).

in several tumours, hence suggesting that the expression of AgNORs could be of help in the diagnosis (prognosis) of hepatic tumours (11), of neoplasias of the cervix (12), endometrium (13-15), urinary bladder (16-17), and meningiomas and astrocytomas (18), squamous neoplasms (19), melanotic lesions of the skin (20) and malignancies of lymphoid tissue (10,21).

Different proposals have been made for the standarizing the evaluation of the AgNOR reaction. the counting of AgNOR particles (8) and the morphometric assessment of the mean area of AgNORs per cell (22) being the basis of all of them. Based on the fact that a progressive increase in both the mean area and numbers of AgNORs taken place as the cell cycle progresses from the G0-G1 to the early - mid and late - mid S-phase (2). a different model for the evaluation of AgNORs has been proposed (3). This model system, based on direct calculation under the light microscope of the proportion of cells with high or low contents of AgNORs, has been used to obtain an approximation to the growth fraction in rat thymic sections (3) and proved to be appropriate to obtain an estimate of the proliferative activity in human biopsy samples of lymphoid tissue (4).

Taking advantage of this model in which two cell populations (type I and type II cells) are identified according to their AgNOR contents, we thougth that measurent of the AgNOR quantity per cell in either of the two groups of cells might provide new insights into the relationship between AgNOR expression and either malignancy or variations in cell cycle parameters. The present study was carried out on that assumption.

In our study, type I cells from the different entities showed a similar expression of AgNORs. In type II cells, the AgNOR content was similar in RLN and NHL. Consequently, AgNOR expression is cannot be said to differ in benign and malignant entities. The higher AgNOR content found in type II cells from HD as compared to that found in the other two groups should be attributable to the cytological characteristics of this kind of entity.

These results further support the hypothesis that changes in the expression of AgNORs, as assessed in tissue sections, are mostly linked to cell proliferation, such changes mainly being due to the variation of the relative numbers of resting (type I cells) and proliferating cells (type II cells) rather than to modifications of the mean AgNOR content of individual cells.

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## RESUMEN

Se piensa que el análisis morfométrico del contenido en AgNORs en células previamente identificadas de forma directa por el observador como con un contenido bajo (célula tipo I) o alto de AgNORs (células tipo II) podría ayudar a esclarecer la relación entre las modificaciones en la expresión de los AgNORs y la malignidad o agresividad biológica de una lesión. Al utilizar este sistema de evaluación de la reacción AgNOR, el resultado se vería libre del sesgo introducido por la proporción de células tipo I y tipo II que, como se ha demostrado previamente, está en relación directa con la fracción de crecimiento.

El estudio se ha realizado en muestras biópsicas de tejido linfoide humano (linfadenopatías reactivas, enfermedad de Hodgkin y linfomas no-Hodgkinianos). Utilizando métodos de análisis de imagen se ha evaluado de forma separada la expresión de los AgNORs (área media de AgNORs por núcleo) en células identificadas de forma subjetiva por el observador como de baja expresión (células tipo I) y células de alta expresión de AgNORs (células tipo II).

El contenido de AgNORs de las células tipo I (baja expresión) fue similar en los tres grupos de muestras estudiados. En lo que concierne a las células tipo II (alta expresión) el contenido de AgNORs fue mayor en las muestras de enfermedad de Hodgkin que en los otros dos grupos. En linfadenopatías reactivas y linfomas no Hodgkin, el contenido de AgNORs de las células tipo II fue aproximadamente 6 veces mayor que el de las células tipo I, siendo esta proporción mayor en las muestras de enfermedad de Hodgkin.

Estos datos refuerzan la hipótesis de que la diferente expresión de los AgNORs en lesiones benignas y malignas es debida a la diferente proporción de células tipo I (células en reposo) y células tipo II (células

proliferantes) más que a modificaciones globales en la expresión de AgNORs de las células de la muestra.

*Palabras clave*: AgNORs. Cuantificación. Tejido linfoide. Biopsia. Proliferación.

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