Nucleolar Structure in Cancer and Its Diagnostic Value*

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ABSTRACT

A modification of the toluidine blue-molybdate stain to demonstrate the internal structure of the nucleolus is described for use in diagnostic cytology. The method demonstrates roughly spherical ribonucleoprotein structures (nucleolini) which are characteristically all the same size in non-neoplastic cells (isoneucleolinosis) but which vary strikingly in neoplastic cells, (anisoneucleolinosis). A diagnosis of malignancy was made independently by nucleolar morphology and by conventional techniques in 96 out of 360 (27 percent) pleural or ascitic effusions. Malignancy was excluded by both methods in 244 of the 285 (68 percent) fluids and was diagnosed by nucleolar morphology only in 16 cases (4 percent). Of these, 11 were known to have malignant neoplasia and eight had definite metastases.

Anisonucleolinar tumor cells with or without isonucleolinar fibroblasts or epithelial cells were cultured in vitro from six out of nine fluids known to contain tumor cells. Only isonucleolinar fibroblasts or epithelial cells grew in vitro from the three other fluids containing malignant cells and from four fluids not containing tumor cells. Thus, the presence of anisonucleolinar cells in cultures of fluids is diagnostic of the presence of tumor cells in the fluid, but the failure to grow such cells is of no diagnostic significance. The internal structure of the nucleolus in body fluids and cultures derived from them is a valuable adjunct to conventional techniques in the diagnosis of malignancy and may identify tumor cells not recognizable by other methods.

Introduction

The internal structure of the nucleolus can be demonstrated by the toluidine blue-molybdate (TBM) method for staining ribonucleoprotein (RNP). One type of RNP occurs in the form of roughly spherical structures which correspond to certain nucleolar vacuoles in the living cell. These vacuoles were well known to the classical cytologists, who termed them nucleolini. The other type of RNP surrounds the nucleolin and is called the body of the nucleolus. The nucleolini are stained meta-
chromatically by the TBM method and correspond to light fibrillar centers in electron micrographs with or without associated peripheral dense fibrillar RNP. The granular ultrastructural component of the nucleolus corresponds to the body of the nucleolus which is stained orthochromatically by the TBM method. The staining method involves the liberation of RNA phosphoryl groups by inactivation of protein-bound amino groups by formaldehyde. The phosphoryl groups of the RNA in the nucleolini form a type of bond with toluidine blue that enables the nucleic acid-dye complex to be polymerized by ammonium molybdate with resultant metachromatic staining. The RNA-dye complex on the body of the nucleolus does not react with molybdate and is therefore stained orthochromatically.

The internal structure of the nucleolus of thousands of preparations of cultured normal diploid cells, aneuploid cells derived from neoplasms, "spontaneously" transformed or transformed by polyoma, SV40 or adenoviruses has been studied during the last nine years. A very clear difference in nucleolar morphology was noted between replicating normal and neoplastic or transformed cells. The nucleolini of replicating normal cells were numerous, small, round regularly distributed and all the same size within a given nucleolus and in all the nucleoli in a nucleus. For this type of morphology the term isonucleolinosis was used. On the contrary, the nucleolini of neoplastic cells were usually large, very unequal in size and often irregularly distributed throughout the nucleolus. This condition has been termed anisornucleolinosis. On the basis of nucleolar morphology, it has been possible to diagnose whether or not a number of unknown cultures were normal or neoplastic. It seemed a logical extension of these in vitro studies to examine clinical material to determine whether or not the internal structure of the nucleolus would be of value in the diagnosis of malignancy. The present report extends our previous studies and concerns the analysis of the internal structure of the nucleolus in 360 pleural and ascitic fluids and a comparison of the diagnoses reached by this method with that made by conventional techniques. The paper also describes the nucleolar structure of the cells that were cultured from malignant and nonmalignant effusions.

Materials and Methods

The toluidine blue molybdate method for the demonstration of internal nucleolar structure by staining of nucleolini is carried out in this manner: Prepare cell pellet in usual way as for Papanicolaou preparations (centrifuge 1,000 rpm for ten minutes). If it is not convenient to process a pleural or ascitic fluid, it may be stored for up to three days in the refrigerator without significant change in nucleolar staining or morphology.

1. Prepare smears of cells in pellet from fluid to be examined as for a blood smear, making two thick smears by smearing slowly and one thin by smearing more quickly. (In most instances the thick smears will be satisfactory but with some bloody or highly proteinaceous fluids; the thin smear will stain best.)

2. Hold the slides horizontally for 30 to 60 seconds to allow cells to settle on the glass. Do not allow to dry.

3. Fix slide, while still wet, cell side down by immersing horizontally into the first fixative—10 percent aqueous trichloroacetic acid. After a few seconds, when the film has stuck to the slide, the slide may then be placed vertically in a rack. Allow to fix for ten minutes at room temperature.

4. Rinse slide gently in a dish of tap water for five seconds.

5. Fix slide for exactly five minutes in formol sublimate at room temperature.
(Freshly prepare nine parts of 6 percent aqueous [saturated] mercuric chloride to one part formaldehyde solution USP which has been stored with marble chips. The time of fixation is important since the staining reaction is dependent upon selective inactivation of protein-bound amino groups by formaldehyde.)

6. Wash slide in running tap water for five minutes. (If it is not convenient to complete the staining procedure, e.g., if the fluid comes in late afternoon, the slides may be stored overnight in distilled water at 4°C.)

7. Immerse slide in Lugol's iodine solutions (USP) for five minutes (5 g I₂ and 10 g KI in 100 ml distilled water).

8. Wash off the iodine solution with tap water and immerse slide in 0.2 M aqueous sodium thiosulphate for five minutes. ( Routinely, a 5 percent solution of the pentahydrate Na₂S₂O₅·5 H₂O is prepared. Replace iodine and thiosulphate solutions weekly.)

9. Wash slide for five minutes in running tap water.

10. Treat slide for one hour at 37°C with deoxyribonuclease (1 mg per dl† in [tris-hydroxymethyl] aminomethane buffer (0.02 M) containing 0.045 M MgCl₂·6 H₂O and 5 mM CaCl₂ anhydrous brought to pH 7.3 by addition of 1 N HCl).

11. Stain for two hours in 15 mg per dl toluidine blue in McIlvaine's buffer with a pH of 3.0 at room temperature. (All samples of toluidine blue certified by the Biological Stain Commission over the last ten years have been tested by this method and found to be satisfactory before certification.) Prepare a 1 percent aqueous stock solution in distilled water and shake overnight. Prepare the staining solution on the day of use by adding 0.75 ml of stock to 49.25 ml of McIlvaine's buffer pH 3.0.)

12. Rinse slide exactly ten seconds in running tap water.

13. Immerse slide in 15 percent aqueous ammonium molybdate for exactly seven minutes at 20 to 25°C. (The molybdate is prepared by dissolving 15 g of (NH₄)₆MoO₄·4 H₂O per dl of demineralized double glass or quartz distilled water. This solution keeps indefinitely. The molybdate reaction does not take place in tap water or demineralized water and is temperature dependent.)

14. Rinse slide with running tap water for exactly ten seconds, drain off excess water by placing the edge of the slide briefly on absorbent paper and dehydrate for ten minutes in tertiary butyl alcohol. (The water rinse is necessary to remove the molybdate which, if it persists, forms a white precipitate with the tertiary butyl alcohol. If washing is too prolonged, some dye may be washed out. Replace butyl alcohol when it develops a bluish color. N.B. Butyl alcohol must be liquid. M.P. = 25.5°C.)

15. Clear slide in xylol and mount in Permount.

Nucleolini will be stained purple in the form of multiple roughly spherical bodies embedded in the body of the nucleolus which is stained pale blue. The structure is revealed by studying the preparations in a green light, e.g., Kodak Wratten filter #58.

Preparations were fixed and stained by the routine Papanicolaou method and cell pellets were embedded in paraffin and stained with hematoxylin and eosin.

For cell culture cells from the pellet in the centrifuge tube were resuspended in Eagle's minimal essential medium containing 10 percent fetal calf serum, glutamine (2 mM) and chlortetracycline (50 µg per ml) and cultured in Blake bottles. In order to study nucleolar structure, cells were seeded at concentrations of 30,000 per ml in “ring” cultures prepared as previously

† Worthington Biochemical, Freehold, NJ 07728.
described\textsuperscript{14} and stained by the TBM method after DNase digestion one day and four days later when diploid normal cultured cells are known to be isonucleolar and aneuploid or neoplastic cells are very anisonucleolar.\textsuperscript{8}

**Results**

**Nucleolar Morphology in Cells of Pleural Effusions and Ascitic Fluids**

Polymorphonuclear cells did not have any nucleolini. Lymphocytes usually had one small nucleolinus or two or three small isonucleolar nucleolini (figures 1 and 2). Macrophages and monocytes had four to ten nucleolini (figure 3). The nucleoli of mesothelial cells usually contain six to 20 moderately sized nucleolini, all about the same size. Since these are frequently in different planes in smear preparations, it is difficult to photograph them. The morphology, however, can be more readily depicted after culture (figure 4).

In contrast, tumor cells have variable numbers of nucleolini which vary considerably in size and some of which are much larger than those of non-malignant cells (figures 5, 6 and 7). Not infrequently, in the tumor cells, there are many nucleolini which are fairly regular in size and one very large nucleolinus (figures 8 and 9). This is a unique feature that is characteristic of malignant cells and is never seen in any other cell. Comparison of preparations stained first by the TBM method and subsequently by the Papanicolaou stain indicates that the nucleolini can often be recognized as small unstained vacuoles.\textsuperscript{6} Figures 1 through 9 were stained by the toluidine molybdate method after digestion with deoxyribonuclease. All photomicrographs: $\times 2,200$.

**Diagnosis by Nucleolar Morphology and by Conventional Methods**

A comparison of the diagnoses in 360 body fluids made independently by nucleolar morphology and in Papanicolaou preparations and in cell blocks is shown in table I. It should be emphasized that the diagnoses were made by two different observers each of which was unaware of the diagnosis made by the other. A positive diagnosis of malignancy was made by all methods in 96 cases (27 percent) and a negative diagnosis in 244 (68 percent). Four cases (<1 percent) were diagnosed by conventional methods and were not recognized by nucleolar morphology. Sixteen cases had definite anisonucleolar cells and were, therefore, positive by their nucleolar morphology and negative by conventional methods. As previously reported,\textsuperscript{6} eight out of 11 were known to have carcinoma and five of these had definite metastases. Three of the remaining five cases had metastasizing carcinoma of breast or lung. One was suspected of having a tumor of the lung and the other had no known malignancy. In summary, 11 out of the 16 cases diagnosed as positive by nucleolar morphology and negative by other methods were
known to have carcinoma and eight of these had metastases.

**CELL CULTURE OF BODY FLUIDS**

The results of successful *in vitro* cultures of the fluids are summarized in table II. Anisonucleolar epithelial cells were grown from six of the nine fluids containing tumor cells (figures 7, 8 and 9). In four instances, these were associated with isonucleolar fibroblasts, in one case with isonucleolar epithelial cells (figure 4) and in only one...
case a pure culture of anisonucleolar tumor cells grew out. Isonucleolar fibroblasts were cultured from three out of four non-malignant effusions, and isonucleolar epithelial cells cultured (figure 4) from the fourth.

Discussion

The results clearly indicate the value of the internal structure of the nucleolus in the diagnosis of malignancy in body fluids. The fact that a positive diagnosis was made only by the TBM method on 11 (3.1 percent) of the fluids from patients known to have carcinoma suggests that the method may be more sensitive than conventional procedures. It is well known that there is often difficulty in the diagnosis of neoplastic cells, particularly when they occur singly in fluids.\textsuperscript{12,13} Nucleolar morphology is very useful in such instances and, in particular, the finding of one cell with one large nucleolinus and many small ones is sufficient to establish a definite diagnosis (figures 8 and 9). At least some of the prominent vacuolation of the nucleoli noted to be a feature of malignant cells\textsuperscript{9} are probably due to the presence of large nucleolini in anisonucleolar cells.

The in vitro studies indicate that the growth of anisonucleolar cells can be taken as diagnostic of malignancy in a body fluid (table II). However, only isonucleolar cells grew out from two fluids containing tumor cells (table II) so that the absence of anisonucleolar cells in a culture cannot be regarded as exclusive of malignancy. It should also be noted that isonucleolar cells grew out along with the anisonucleolar tumor cells in all but one instance. It is, therefore, important to examine the nucleolar morphology of cultures

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<th>TABLE I</th>
<th>Diagnosis of Malignant Cells in 360 Pleural and Ascitic Effusions</th>
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<tr>
<td>Papanicolaou</td>
<td>Nucleolar Morphology</td>
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<tr>
<td>+    +</td>
<td>96</td>
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<td>-    -</td>
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of body fluids since many of the cultured cells are derived from non-malignant cells in the fluid. In no instance did anisonucleolar cells grow from the non-malignant effusion. Thus, cell culture can be a useful adjunct to the direct study of smears in the diagnosis of malignancy in body fluids. Furthermore, the cells in culture spread out on the glass so that the nucleolinar morphology is more readily seen (figures 4, 7, 8 and 9).

It might be noted that nucleolinar morphology can only be studied in smears and monolayers of cells and not in sections. In the process of sectioning the nucleoli and nucleolini would be cut in various planes with the result that nucleolini of identical size would appear to be unequal and a mistaken diagnosis of anisonucleolinosis would be made.

### Summary

A modified toluidine blue molybdate stain is described for use in the diagnosis of malignant cells in body fluids. The method demonstrates roughly spherical ribonucleoprotein structures (nucleolini) in the nucleolus which are characteristically all the same size in non-neoplastic cells (isonucleolinosis) but which vary strikingly in size in neoplastic cells (anisonucleolinosis). A diagnosis of malignancy was made independently by nucleolar morphology and by conventional techniques in 96 out of 360 pleural or ascitic effusions. Malignancy was excluded by both methods in 244 of the 360 fluids and was diagnosed by nucleolar morphology alone in 16 cases (4 percent). Of these, 11 were known to have carcinoma and eight to have metastases. The internal structure of the nucleolus would appear to be a reliable method for the diagnosis of malignancy and can identify malignant cells not detectable by other methods.

### References

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9. **Miyake, T., Yamamoto, Y., Ariyoshi, J.,**


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Plan to attend the 25th Anniversary Meeting of the ASSOCIATION OF CLINICAL SCIENTISTS in Philadelphia, PA on November 8, 9 and 10, 1974

Applied Seminar on the Laboratory Diagnosis of Skeletal, Muscular and Nervous Disorders