A Method of Staining Nucleoli of Cells in Fresh Benign and Malignant Tissues*

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INTRODUCTION

With the increasing interest in the diagnosis of malignant cells in various exudates and body fluids, a method demonstrating certain nucleolar changes in considerable detail may be of value. It is the purpose of this paper to present such a method and to discuss briefly the nucleolar changes in benign and malignant tissues and also to point out the potential applicability of this method as an aid in the diagnosis of cancer.

The azure C method is based on the observation that nucleoli of cells of fresh tissues when exposed to certain dyes, stain electively. Naegeli (17, 18) states that the nucleoli of lymphocytes and myeloblasts often stain well with supravital technics as described by Pappenheim, Nakanishi, Sabrazes, Schilling-Torgau and Cesaris-Demel. These methods utilize methylene blue, brilliant cresyl blue or Giemsa stain. Quensel (21, 22), in particular, has studied the nucleoli of malignant cells in pleural and ascitic fluids using a staining mixture of methylene-blue-cadmium and Sudan III-cadmium. He states that with his method the nucleoli stain better than with any other method. MacCarty (13, 14), and MacCarty and Haumeder (15), in extensive work on the nucleoli and the nuclear-nucleolar ratio of benign and malignant cells, recommend Terry's or Unnas' polychrome methylene blue for the staining of these bodies in frozen sections. MacCarty states that the nucleolus may be better demonstrated in fresh tissues than in fixed tissues. Guzman (7, 8), in a study of the nucleoli of lymphocytes and monocytes of the peripheral blood, developed a method utilizing diluted Leishman or Giemsa stain. Von Haam and Alexander (25, 26), in their work on the cytology of malignant tumors, developed a method of staining nucleoli of fresh unfixed cells in suspension, with high dilutions (1:10,000) of toluidine blue.

In most of the methods mentioned above, methylene blue or derivatives of methylene blue have been used to produce the elective staining of nucleoli. No systematic studies have been done on the homologues of methylene blue as to their ability to stain nucleoli. In the development of the azure C method this was done. Methylene blue, azure A, azure B, azure C, and thionin were studied for their staining qualities for nucleoli in many fresh benign and malignant tissues. It was found that azure C gave the best results in that the nucleoli showed a sharper outline and increased metachromatic effects, and there was less tendency of the nucleus to be overstained as compared to the other dyes. Azure B gave almost as good results. Azure C, or mono-methyl thionin, was first prepared by Holmes and French (10) in 1926, by the oxidation of methylene blue under acid conditions.

TECHNIC

Preparation of the stained slides.—Microslides containing a fine precipitate of azure C are prepared as follows: Solutions of azure C in 0.25 per cent and 0.50 per cent concentrations in absolute methyl alcohol are prepared. A drop of one of these solutions is placed on a clean microslide and quickly covered with another slide. The stain diffuses between the slides. Excess stain is removed from the slides. The coverslip is rimmed with vaseline and quickly covered with another slide. The stain diffuses between the slides. Excess stain is removed from the edges with a cloth. The slides are then pulled apart with a rapid sliding motion in the long axis of the slides. The alcohol rapidly dries, leaving a fine precipitate of dye. After marking one corner of the slides with a wax pencil for identification of the stained surface, they are stored until required. Slides are made with both concentrations of the dye.

Technic for tissues—A new cut is made in fresh tissue and the surface lightly scraped with a scalpel. The small amount of tissue thus obtained is stirred into a drop of fresh human blood serum which has been previously placed on a coverslip. The tip of the scalpel is used for stirring. The coverslip is inverted over the stained slide. This results in diffusion of the cell-suspension between the two surfaces. Pressure on the coverslip is avoided. The coverslip is rimmed with vaseline to

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prevent drying. The nucleoli usually stain within a few minutes. The slide is examined under the oil immersion lens immediately after preparation. Certain precautions are necessary to obtain good results. The drop of serum should be very small so that with application of the coverslip to the slide, a very thin film of the cell suspension, which does not quite reach the edges of the coverslip, is obtained. The amount of tissue, likewise, should be very small so that the cells are somewhat flattened and no more than one cell layer in thickness. Clumps of cells are to be avoided, since the cells within the interior of the clumps do not stain. Stained slides prepared with the 0.50 per cent concentration of the dye are used for tissue. If the nucleoli do not stain, or if they stain poorly, the usual faults are: too much serum, too heavy a suspension of cells for the amount of dye, too light a concentration of dye on the slide or insufficient interval of time for staining. If the nucleus over-stains, the usual faults are: too heavy a concentration of dye on the slide for the number of cells, or autolysed tissue. For best results, tissues should be used immediately after removal from the body, since the differential staining between nucleolus and nucleus is not as distinct when old tissues are used. The best results are obtained with cells free in fluid, tissues in which the cells have not adhered to the stroma, as found in many carcinomas. The so-called "tumor milk," found on the cut surfaces of carcinomas, is especially suitable for study by this technic. Good results are not obtained with dense tissues in which cells are loosened with difficulty from the stroma. Trauma often results in distortion of the cells, and necrotic areas within a tumor are to be avoided. Several parts of a tumor should be sampled to reduce the possibility of missing the malignant portion of the lesion.

Staining of blood.—The finger is pricked and a small drop of blood is placed in the center of a coverslip which is then inverted over the stained slide. Slides prepared with the 0.25 per cent solution of dye are ordinarily used, but for leukemias a higher concentration of the dye may be required.

Staining of transudates, exudates and aspiration biopsies.—The preparations are made as described above, using the whole fluid or the sediment obtained after centrifugation, with or without dilution of the material with serum depending on its cellularity. The slides prepared with the 0.50 per cent dye concentration are used but it may be necessary to vary the concentration of the dye. In aspiration biopsies, if bits of tissue are obtained, these should be gently rubbed into the drop of serum on the coverslip to free the cells from the stroma. Bone marrow obtained by aspiration need not be mixed with serum, whereas bone marrow obtained at autopsy should be so mixed.

RESULTS

The nucleolus stains a brilliant uniformly dark azure and is in striking contrast to the relatively unstained nucleus. The outline of the nucleolus is sharply delineated; fine strands of nucleolar material may be seen between some nucleoli. Small nucleoli, which are often obliterated by heavily stained blocks of basophilic chromatin of the nucleus in fixed preparations stained with the usual methods, are well seen by this method. Vacuoles within the nucleolus are often noted. No staining distinction between plasmosomes and karyosomes is noted. The nucleus does not stain, or stains a light azure, light green, or light blue, depending on the type of cell. The cytoplasm usually assumes about the same color as the nucleolus, but may not stain or may assume a light azure to dark blue depending on the type of cell. The border often is not seen in cells removed from tissue. Staining takes place in 1 to 15 minutes.

Although the preparations are not permanent they may be kept in fair condition for several days if refrigerated. Degenerative changes vary considerably with the cell type. For example, the monocyte of the peripheral blood degenerates rapidly and there is a tendency for the nucleus to be over-stained, obscuring the 2 to 5 very small nucleoli contained in this cell. In contrast, there is little tendency for the nucleus to be over-stained in malignant cells and the period of profitable observation may last for hours. In general, more immature cells show less degenerative changes than do more mature cells. The cytoplasm undergoes degeneration more rapidly than does the nucleus, and the nucleus more rapidly than the nucleolus. Cells in mitosis are easily recognized. The chromosomal mass takes a light blue stain and the nucleolar fragments continue to take the dark azure stain. Individual chromosomes are not stained. Quensel (22), with his method, states that mitotic figures are not readily stained.

It has been observed in actively metabolizing benign and malignant tissues and particularly in the blood in leukemia, that after once staining, the cells may quickly become decolorized. This phenomenon has also been observed by Simpson (24), using brilliant cresyl blue, and by Quensel (22), using methylene blue. The dye is reduced to a leukobase, probably by enzymatic action of the tissue. The process is reversible and restaining will occur if one

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corner of the coverslip is lifted, permitting the entrance of air to the cells. Of considerable interest is the work of Barron (1), who states that methylene blue, to which azure C is related, stimulates oxygen consumption of normal tissues, having aerobic glycolysis, and of tumors.

A detailed description of specific cells is not indicated here but a brief description of a few common cells found in serous exudates will be given.

The red cells do not take the stain. The reticulum of the reticulocytes takes a light brown stain. The small lymphocyte (Fig. 7) contains usually 1 small round eccentrically placed nucleolus and occasionally 2, but no more than 2, nucleoli. There is a thin rim of azure cytoplasm around the relatively unstained round nucleus. The monocyte of the peripheral blood contains 2 to 5 small nucleoli about half the size of those found in the small lymphocyte. The nucleus is approximately oval in shape, whereas the cytoplasm is relatively abundant and takes the azure stain. The mesothelial cell (Fig. 9) contains usually 1 small round dark azure-staining nucleolus in a round to oval nucleus and a relatively small amount of azure-stained cytoplasm. Macrophages in fluids show an oval to elongated nucleus, abundant cytoplasm that is often vacuolated, and 1 to 2 small oval to round nucleoli. They often contain ingested material and cells. Polymorphonuclear leukocytes may be recognized by the shape of the nucleus, the fine cytoplasmic granules and the absence of the nucleoli. Basophils and mast cells show bright red granules, while in eosinophils, some of the granules have a light azure tint. The plasma cell shows an oval, green-tinted nucleus with usually 1 eccentrically placed small round nucleolus, but it may contain up to 3 nucleoli. The cytoplasm is abundant, light azure and finely vacuolated. Normoblasts show a pale blue stained nucleus. Fat does not stain. Melanin pigment of malignant melanoma stains dark blue.

DISCUSSION

Advantages and disadvantages of the method.—The advantages of the method are (a) nucleoli may be demonstrated with detail not ordinarily seen in other types of preparations using fixed and embedded tissues; (b) rapidity of the method; (c) applicability to exudates, aspiration biopsies and other types of fluid or semifluid tissues; (d) complete cells are seen and the total number and complete outline of the nucleoli may be noted, in distinction to sectioned tissues; (e) certain crystals such as Charcot-Leyden crystals, uric acid crystals or cholesterol crystals are seen (they are usually dissolved when other technics are used); (f) artefacts produced by fixation and embedding are avoided.

The disadvantages are: (a) impermanence of the preparations; (b) degenerative changes occurring in unfixed tissues with the production of artefacts; (c) in the study of malignancy, the failure to see structure of the tumor, its relation to stroma or its invasive properties; (d) sampling of only a small part of the tissue with the attendant possibility of missing the lesion. This method, however, complements other methods and should be used in conjunction with other histopathological technics.

The nucleoli of malignant cells.—The nucleolar changes of malignant cells have been studied in particular by Pianese (20), Quensel (21, 22), MacCarty (13, 14), MacCarty and Haumeder (15), Fidler (5), Castren (2), Saxon (23), Karp (11), Zadek (27), and von Haam and Alexander (26). Among the nucleolar changes in malignant cells described by these authors are: (a) increase in size of the nucleoli out of proportion to the increase in size of the nucleus; (b) irregularity in the shape of the nucleolus; (c) increase in the number of nucleoli; (d) variation in the number of nucleoli. There are two other features which the author has observed in some malignant cells: (a) the presence of fine nucleolar strands between nucleoli, morphological evidence of nucleolar division; (b) loss of polarity of nucleoli (the nucleoli are arranged at right angles to the long axis of an elongated nucleus). None of these changes are pathognomonic of malignancy. Von Haam and Alexander (26) state "neither size, shape or number of nucleoli represent any common characteristic for malignant cells." Quensel (22) states, "by my method peculiar changes in the nucleoli of malignant cells in serous exudates are so regularly found that when these changes are marked, they represent an entirely characteristic sign of malignancy." He further states that all malignant cells may not show these nucleolar changes. At present it is the consensus that there is no common pathognomonic morphologic feature of the malignant cell.

Although the nucleolar changes described above are not pathognomonic of malignancy, they are of great help in arriving at a diagnosis. It has been observed that most non-neoplastic tissues and benign tumors contain from 1 to 3 small round nucleoli. However, Von Haam and Alexander found large nucleoli in the corpus luteum cells of the ovary, and Ferreira (4) has noticed similar changes in benign tissues. He observed in the cells of the fetus, normal liver, membrana granulosa of the ovary and in exophthalmic goiter, an
increase in the area of the nucleolus and a decrease in the nuclear-nucleolar ratio which approximate those of cancer. In the present study, some of the nucleolar changes already mentioned have been found in rapidly growing benign tissues such as the megaloblasts of pernicious anemia, abnormal lymphocytes in infectious mononucleosis, rapidly proliferating endometrium, rapidly growing fetal tissues and fibroblasts of young granulation tissue. On the other hand, small, round, apparently benign nucleoli have been found in lymphosarcoma of the lymphocytic type, in small cell bronchogenic carcinoma and in neuroblastoma. Ludwig (12) summarizes the matter when he states, "actively growing cells are characterized by their large nucleoli, and by the fragmentation and division of same. In the adult, nucleoli of cells undergoing active metabolism are relatively large; those in the 'resting' condition relatively small." It is highly probable then that the nucleolar changes herein described may be found in any rapidly proliferating tissue, of which malignant tissue is a notable example.

**Application of the azure C method.**—The usual histopathologic methods are adequate in the diagnosis of malignancy in tissue and are to be preferred to scraping of tumors, in which only individual cells or groups of cells are found. Even here, however, more information of the finer cytology may be obtained by means of the azure C stain. On the other hand, considerable difficulty has long been experienced in the diagnosis of malignancy in the individual cells found in various body fluids and exudates. The sediments of the fluids are embedded and sectioned with difficulty. The alternative methods of studying these cells are by the preparation of smears stained with hematoxylin and eosin, or Papanicolaou's technic (19), or the method of Dudgeon and Patrick (3), or by embedding and sectioning the sediment according to the method described by Mandelbaum (16) and Zemansky (28). Although a fairly high degree of accuracy in diagnosis may be obtained by these methods, difficulty is still experienced in the separation of macrophages and mesothelial cells from some malignant cells. In this regard, Foot (6) states, "mesothelial pleural, pericardial and peritoneal covering cells present the chief obstacle in the way of successful diagnosis as they are readily confused with tumor cells on account of their large size and prominent nucleus." It is in the differentiation of these cells from malignant cells that the azure C method is particularly applicable. With the azure C method, the mesothelial cell usually contains one small round nucleolus, as does the macrophage. In sharp contrast, many malignant cells contain large, bizarre-shaped and multiple nucleoli. The absence of these changes does not rule out malignancy completely, since the tumor must be on a cavity surface and cells must be sloughing into the cavity in order that the malignant cells may be found in the fluid. This has been previously emphasized by Helwig (9). The nucleolar changes described above should be extensive, and should be present in many cells, to be of aid in the diagnosis of malignancy.

At present 200 tissues have been studied at the Naval Medical School and high diagnostic accuracy has been achieved. As more data are collected they will be the subject of a later report.

The azure C method may be used for a more accurate description of nucleoli of cells. In general, nucleoli are rather vaguely described chiefly because fixed tissues and fixed smears of tissues are employed. In a fixed preparation there is precipitation of chromatin on the inner wall of the nuclear membrane in the lining network and on the outer surface of the nucleolus. This precipitation of nuclear material obscures the outline of the nucleolus which gives a false picture of size, obliterates fine nucleolar strands and may entirely hide some of the smaller nucleoli.

In frozen sections cellular detail is not well seen and it is necessary to wait 24 hours or so for the paraffin sections. With the azure C method, this difficulty may be partly obviated since considerable cellular detail may be seen at the time the frozen section is done. The azure C slide may be read often within 5 minutes, and in conjunction with the frozen section.

**SUMMARY**

1. A method is presented by which the nucleoli of cells of fresh benign and malignant tissues may be observed in great detail. Cell suspensions in serum are prepared on a coverslip which is then inverted over a slide containing a fine precipitate of azure C dye. The nucleoli stain clearly and distinctly, permitting contrast between the nucleus and nucleolus. Advantages as well as disadvantages of the methods are described. A brief description of a few common cells as they appear with this method is given.

2. The nucleolar changes in malignant tissues and fast growing tissues are compared with those of benign tissues.

3. The value of the method as an aid in the differential diagnosis of benign and malignant cells in serous effusions is discussed.
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REFERENCES

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Fig. 1.—Cell of malignant melanoma. The dark irregularly shaped central masses represent nucleoli.
Fig. 2.—Cell of malignant melanoma.
Fig. 3.—Cells of adenocarcinoma of rectum.
Fig. 4.—Cell of transitional cell carcinoma of bladder. All nucleoli are not in focus. All photographs at a magnitude of 900 diameters.
Figs. 1-4
DESCRIPTION OF FIGURES 5 TO 10

**FIG. 5.**—Normal cell from bronchiolar epithelium. Note the small round nucleoli.

**FIG. 6.**—Malignant cell from metastatic carcinoma to bone. Aspiration biopsy of lesion in scapula. Note the bizarre shape of the nucleoli and the fine nucleolar strands.

**FIG. 7.**—Normal mature small lymphocyte. Note the single small round eccentrically placed nucleolus. One cell is not in focus.

**FIG. 8.**—Megaloblast from a patient with pernicious anemia. Note the nucleolar strand, and the bizarre shape of the nucleolus present in a benign cell.

**FIG. 9.**—Mesothelial cells from visceral pleura of lung.

**FIG. 10.**—Malignant cell of adenocarcinoma of rectum, metastatic to peritoneum. In ascitic fluid.

All photographs at a magnification of 900 diameters.
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