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Some Cytochemical Observations on the Nucleolus of Mouse Fibroblast Treated with Dilute Culture Medium[1)]

By

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(With 3 Text-figures)

It has widely been accepted that the nucleolus is a heterogeneous mass consisting of two morphologically different constituents, filamentous structure or nucleolonema, and amorphous matrix or pars amorph (Bernhard *et al.* 1955, Borysko and Bang 1951, Davies 1952, Denues and Mottram 1955, Estable and Sotelo 1951, 1555). It has also been well known that numerous agents, viz., hypo- and hypertonicity, pH, heat, organic compounds, x-irradiations and so on, exert influence upon the nucleolar morphology (cf. reviews by Vincent 1955, and Hughes 1952a). Hughes (1952b) and Lettré and Siebs (1954) observed that the nucleolus of the chick fibroblast was disintegrated by the action of adenosine into two components; fibrous structure and fine drops adhering to the former. Further, Lettré and Siebs (1954) found that the fibrous material was stained with both Feulgen's stain and methyl green, and the fine drops with pyronine. Based on these results, they considered the former to be nucleolonema and the latter to be pars amorph. In the course of chemical analysis of isolated liver nucleoli, Monty *et al.* (1956) ascertained that the percentage of DNA in the isolated nucleoli was higher than that in the chromosomal fraction. Further, they demonstrated the presence of DNA in the nucleolus by the application of histochemical methods.

The present author has been making a morphological and cytochemical study of the nucleolus of tumor cells; he has reached the conclusion that the real feature of the nucleolus can not be well understood unless any possible close relationship between the nucleolus and its associated chromatin is analysed (Hori 1956 a,b). With this view in mind, the present study was undertaken in order to inquire into the real site of the cytochemical properties of the nucleolus by removing the associated chromatin from the nucleolus.

Pieces of hearts taken from mouse embryos were planted on coverslips in clots, composed of equal parts of chicken plasma and 50 % chick embryo extract in Gey's solution. They were cultured by the usual roller tube method with 3 ml of culture medium containing 1 part of 50 % chick embryo extract, 1 part of horse serum, 3 parts of Gey's

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solution, about 300 units of penicillin G, and about 300 μg of streptomycin. Incubation was made at 37°C with tube-rotation at 15 times per hour. After 4 to 5 days of incubation, the cultures were immersed for 30 minutes at 37°C in a dilute medium comprised of 2 parts of distilled water and 1 part of culture medium. After this treatment, the coverslips were fixed with Carnoy's solution (6, 3, 1) for 1 hour and then washed thoroughly with 95 % alcohol. Various staining methods were applied to pretreated and non-treated cultures; they are : 1) the Feulgen reaction by Stowell's modification (1945), 2) the methyl green-pyronine[1] staining according to Brachet (1940), 3) light green staining (0.5% light green solution in 95% alcohol), 4) routine staining by Delafield's haematoxylin, 5) Baker's acid haematin test for phospholipids (1946), and 6) lipid staining by Sudan black B according to Lison's prescription (1936).

The nuclei of normal mouse fibroblasts contained a variable number of nucleoli. In the Feulgen preparations a few prominent heterochromatinic granules were found adhering to the nucleolus which is colorless and surrounded by a thin layer of chromatinic substance (Fig. 1) When methyl green-pyronine staining was applied to cells, the heterochromatinic granules were stained bright green and the nucleoli pink, while the nuclear chromatinic granules and threads were blue or bluish green. The nucleolus, fixed with Carnoy's solution and stained with haematoxylin, was not of homogeneous structure. Slightly stained parts resembling vacuoli were often visible within the nucleolus. This heterogenous structure of the nucleolus was also revealed by Baker's test as well as by methyl green-pyronine staining. The pretreatment of cultures with a dilute medium gave rise to partial dissolution of the nucleolus, and also to almost complete diffusion of chromatinic substance. The nucleolus-associated heterochromatin showed similar diffusion through this treatment. As a result the nucleolus appeared without attached chromatinic substance. Observations with the phase microscope revealed a filamentous structure in certain nucleoli together with various heterogenous features

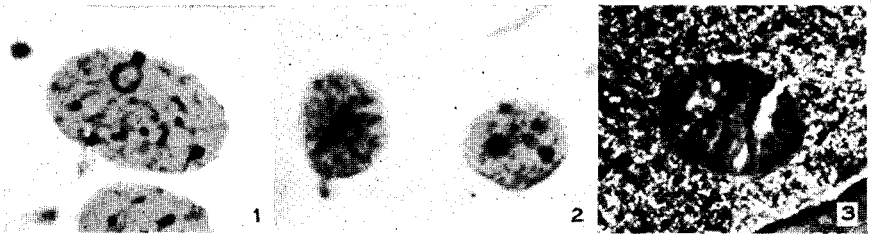

Fig. 1. A normal fibroblast stained by the Feulgen reagent. × 1600.

Fig. 2. Fibroblasts treated with dilute medium and stained by the Feulgen reagent. × 1600.

Fig. 3. A living fibroblast under the treated condition. Phase contrast. × 720.

1) Both methyl green and pyronine were purified after Shibatani's method (1949, 1950) prior to the preparation of the Unna-Pappenheim combination.

Cytochemical and tinctorial properties of the nuclear

	Feulgen		Methyl green-pyronine	
	normal	treated	normal	treated
Chromatin, granules & threads	positive	diffused	green or bluish green	diffused
nucleolus	negative	positive	pink	pale purple
nucleolus-associated chromatin	positive	diffused	bright green	diffused

* Nucleoli were bright green when treated with 1N HCl for 10 minutes

in some others (Fig. 3). It seems probable that the above changes have occurred due to the dissolution of a certain component of the nucleolus. In any case, the nucleolus itself was only slightly affected by the treatment. However, certain tinctorial properties of the nucleolus were influenced. Its stainability by pyronine or haematoxylin was fairly reduced. When the methyl green-pyronine method was applied to the treated culture, the nucleolus was stained faintly green, and pyronine color was superimposed on faint green color. After digestion with ribonuclease the pyronine color was considerably reduced, though not completely and as a result the nucleolus stained pale purple. In contrast to a such reduction in stainability, purple-red color became evident in the treated nucleolus following the Feulgen reaction (Fig. 2). Here, noteworthy is the fact that the positive reaction has appeared being restricted to the surface or the outer layer of the nucleolus. In control preparations, which were not hydrolysed by 1N HCl and stained with Schiff's reagent, the nucleolus always showed negative reaction to the dye; on this basis it is reasonable to see that the positive reaction here obtained indicates the presence of DNA in the nucleolus. However, there is a possibility of the redistribution of DNA to the nucleolus from the nucleoplasm through the diffusion of chromatinic substances after treatment. The nucleoli, both treated and nontreated, were stained very slightly with light green. But, when the cells were hydrolysed with 1N HCl at 60°C for 10 minutes prior to staining, bright green color developed in the nucleolus after staining. This phenomenon seems to indicate the possible presence of nonhiston protein in the nucleolus (Kaufmann et al. 1951). Further, it seems probable that the protein in question can not be dissolved away from the nucleolus through the treatment with a dilute medium. Sudan black B could stain neither the normal nor the treated nucleolus. The results of cytochemical tests made on the nuclear components of the mouse fibroblasts are as summarized in Table 1.

The experimental modification of nucleolar morphology has long been carried out by many workers. It is necessary, as it seems to the author, to pay attention to the nucleolus-associated chromatin when cytochemical tests on DNA are to be

components under normal and treated conditions

Haematoxylin		Light green		Acid haematein		Sudan black B normal & treated
normal	treated	normal	treated	normal	treated	
++	+	+	+	−	−	−
++	+	±(++)*	±(++)*	++	++	−
++	+	+	+	−	−	−

at 60°C.

applied to the nucleolus under modified conditions, because the chromatinic substance in question would cause a shift in certain cytochemical properties of the treated nucleolus. In the present study, the author has attempted to separate the chromatin from the nucleolus by the use of dilute medium. The separation seemed to be successfully made, since the dissolution of the chromatin took place. On the other hand, the treatment with dilute medium partly converted the nature of the nucleolus into Feulgen-positive. As already mentioned, Lettré and Siebs (1954) and Monty et al. (1956) were successful to demonstrate the Feulgen-positive nucleolus. But, they studied the nucleoli under treated conditions as in the present study. Thus, the question why the reactivity of the nucleolus to the Feulgen's stain is shifted by some treatments remains unanswered.

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It is a pleasure to dedicate this article to Professor Tohru Uchida in celebration of his 60th birthday.

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