Nile Blue Inducible Fluorescence of Tumour Cells

A. L. BASTOS and DANTE MARQUES

Serviço de Patologia Celular Calouste Gulbenkian, Instituto Português de Oncologia Francisco Gentil, Lisboa 4, Portugal

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An oxazine dye, Nile Blue sulfate, induces a fluorescent reaction in cytoplasmic granules (NBIFG) of living or fixed tumour cells in the same manner as reported before for Thiazine dyes.

The NBIFG correspond to phase contrast positive granules when cells are viewed by phase contrast microscopy. Fluorescence disappears from NBIFG in a matter of 2—4 days and the bodies turn deep blue. These granules have a succinate dehydrogenase (SDG) activity and are negative for acid phosphatase, peroxidasic activity and porphyrin.

The cytological findings support the assumption that Nile Blue sulfate forms a salt linkage with unsaturated fatty acids of NBIFG which also show an oxi-reductive activity. The molecular nature of the fluorophore(s) is (are) unknown.

We have previously shown that thiazine dyes can induce a fluorescent reaction in cytoplasmic granules of tumour cells when freshly stained by these dyes and examined by fluorescence microscopy. The fluorescent reaction is called primary when the granules are visualized immediately after the staining; secondary, when the granules become fluorescent only after exposure of the cells to light. The primary reaction is observed only with a particular batch of Toluidine Blue and the secondary reaction with all Thiazine dyes so far tested, including samples obtained from several manufacturers.

The dye inducible fluorescent (DIF) granules are refractile, which makes their microscopic recognition easy (i.e. Interference microscopy); cytochemical reactions for lipids (Oil Red O, Sudan Black and reduction of osmium tetroxide) are positive.

Sudanophilia and osmiophilia usually indicate the presence of unsaturated fatty acids. Since the presence of these highly oxidizable lipids could help to explain the cytochemical nature of the DIF reaction, Lillie's Nile Blue procedure was used to demonstrate unsaturated fatty acids.

The observations now reported show that Nile Blue sulfate (samples from several manufacturers) also gave the DIF reaction in the same granules stained by Thiazine dyes, both in living and fixed cells.

Requests for reprints should be sent to Dr. A. L. BASTOS, Servio de Patologia Celular Calouste Gulbenkian, Instituto Português de Oncologia Francisco Gentil, Lisboa 4, Portugal.

Material and Methods

Tumour Cells

The staining procedures (supravital and in vivo staining) were carried out using transplantable tumour cells of mice, Sarcoma 37 tumour cells and Ehrlich tumour cells growing in ascitic form. Cells were obtained from tumour-bearing mice 5 to 10 days after cell transplantation. The experiments were carried out while the tumour was maintained during 378 consecutive passages.

Nile Blue Staining

A modification of Lillie's technique for demonstration of fatty acids was used as follows:

1. Fix tumour cells in 4% formaldehyde saline at 4°C for 11/2 hours.
2. Remove the excess fixative by cell centrifugation for 6—10 min at 1000 rpm.
3. Resuspend the cells in an aqueous solution of Nile Blue (0.005% w/v) and stain for 30 min.
4. Mount the cell sediment in glycerol.
5. Examine by fluorescence microscopy.

Fixatives

To study the effect of fixation the following fluids were used: Neutral formalin at 10% and 15%, Carnoy's fluid, Alcohol-Ether, 2.5% glutaraldehyde in cacodylate HCl buffer.

Control of pH, dye concentration, temperature and staining time

a) pH: The cells were stained in Nile Blue solution at pH 0.9—12. The pH was buffer adjusted with NaOH (saturated solution). Above pH 9 the colour of the solution changes from blue to pink and precipitates.

b) Dye concentration and temperature: The dye concentration in the staining solution
was varied in tenfold dilutions from 0.5% to 0.00005% (w/v). The ratio of the cell volume to the volume of staining solution was 1:10. The temperature of the staining solution ranged from 0 °C to 100 °C.

c) Staining time: The staining times used were as follows: 0', 1', 5', 10', 15', 30', 60', 2 hrs., 12 hrs., 24 hrs., 48 hrs.

**Nile Blue Compounds tested**

Nile Blue Sulfate [C.I. (913)51180] British Drug Houses, Poole, England, Batch N. 2868090; Matheson Coleman & Bell, Norwood, Ohio, USA, Batch N. 481208; Chroma-Gesellschaft Schmid & Co., Stuttgart, Germany, Batch unknown. All samples gave the fluorescent reaction.

**Microscopic equipment**

A Wild M20 microscope with a dual illumination system for fluorescence, bright field and phase contrast was used. It was fitted with Xenon lamp XBO 150w for fluorescence and a 12 V/100 w quartziodine lamp for bright field and phase contrast.

**Fluorescence**

For Ultraviolet excitation — (c. 365 nm) the filter set consisted of a heat-absorbing filter KG 1², UG 1 excitation filter ³. Red absorbing filter BG 38 ⁴ and GG 13 ¹ as the barrier filter. For Blue light excitation — (c. 400 nm) a BG 12 ³ excitation filter and OG 1 ⁵ barrier filter were used.

**Cytochemistry**

In order to investigate the biological nature of DIF granules, we employed the GOMORI lead phosphate method ⁴ for lysosomes, peroxidactic activity in peroxisomes, using 3 - 3' diamino benzidine (DBA) at pH 9 ⁶, succinodehydrogenase activity in mitochondria using a non-lipid soluble formazan forming salt-Nitro-BT ⁸ and perchloric acid-mercapto ethylamine UV method for porphyrins ⁷.

The above cytochemical preparations were then stained by Nile Blue sulfate to demonstrate the presence of DIF granules and slides examined both by light and fluorescence microscopy.

**Results**

The fluorescence microscopic appearance of vitally and supravitally stained and fixed tumour cells are shown in Figs. 1, 2* and 3. The Nile Blue indu-
Formalin fixed Sarcoma 37 tumour cells stained by Nile Blue. Bright yellow emission of NBIFG with blue light excitation. 800 x.

cible fluorescent (NBIF) granules are identical to those shown in Fig. 1 A, when tumour cells were stained fresh with a particular batch of Toluidine blue.

The fluorescent reaction is visualized immediately in living and formalin-fixed tumour cells provided that the latter are examined with blue light excitation (c. 400 nm). When these fixed cells are examined with UV light (c. 360 nm), no fluorescence is seen. A fluorescent reaction develops in these preparations in about 1 — 2 min, when the cells are exposed to blue light, UV light or — in a shorter time — to a strong white light source.

The conditions for light exposure are the same as those used for fluorescence microscopic observations. After light exposure the granules are readily seen when preparations are observed under UV excitation. The colour emission (green — UV excitation) of these light exposed formalin-fixed Nile Blue stained preparations is identical to that obtained from vital and supravital staining.

Under bright field microscopic conditions, the granules are practically invisible and colourless using fixed cell preparations, but are really detected using phase contrast equipment. Figs. 4 a and 4 b show the same cell under bright light and phase contrast, using an automatic photographic exposure.

Figs. 5 a and 5 b serve to demonstrate that the phase positive granules are fluorescent, as the same cells were photographed using phase contrast and fluorescence microscopy. Graph 1 represents the correlation of phase-positive granules per cell to fluorescent granules per cell.

A constant observation of Nile Blue stained (pH 5) fixed cells is the disappearance of the fluorescence in a matter of 2 — 4 days with the granules turning deep blue.

Visual control of the preparations obtained under different staining conditions (Dye concentration, temperature and staining time) showed that fluorescence of NBIF granules (Excitation at 400) was not affected by these variables.

Figs. 7 to 10 illustrate simultaneously acid phosphatase, peroxidactic activity, succinodehydrogenase, porphyrin and NBIF bodies in the same preparations. These NBIF granules exhibit an associated SDG activity (Figs. 9 a — 9 b) with demonstrable fluorescence in all cytochemical double stained preparations. A clear differentiation is seen between red porphyrin fluorescence and yellow emission from the NBIF granules (Figs. 10 a — 10 b). No acid phosphatase or peroxidactic activities are detected in NBIF bodies.

Discussion

The microscopic data presented suggest that the granules which fluoresce are identical to those previously discussed in relation to Thiazine Dyes.

Since lipids have been cytochemically demonstrated in these Nile Blue fluorescent granules, it is interesting to note the good correlation between counting of phase contrast granules per cell and fluorescent granules per cell. It is to be noted that Nile Blue was initially used to demonstrate the presence of fatty acids. DUNNIGAN suggests a salt linkage between the oxazine base and fatty acids which is not dependent on critical staining conditions. If this is so an acid-base reaction could explain that NBIF granules can be stained at 0 °C in a few seconds and at dye concentrations of 0.00005%.

Although in our earlier papers we stated that fluorescent cytoplasmic granules with affinity for basic dyes might be lysosomes, cytochemical studies reported elsewhere in a preliminary form and above suggest that this is not the case. When double staining techniques are used (Nile Blue for DIF granules and Gomori acid phosphatase for lysosomes) the DIF granules show no acid phosphatase.
In fact the DIF granules show succinate dehydrogenase activity, suggesting that they may be related to mitochondria. Perhaps the appearance of the Chromodye reflects the existence of an oxidation-reduction process.

Nile Blue has been extensively used in Cytchemistry as an indicator for acidic and neutral lipids. The fact that the Chromodye observed is either blue or pink seems to indicate the presence of both these components. It is interesting to note that the light induced fluorescence, for observation in UV, can be studied qualitatively as well as quantitatively using microfluorometric equipment as mentioned elsewhere.

5 K-I. Hirai, J. Histochem. Cytochem. 17 (9), 585 [1969].
9 M. G. Dunnigan, Stain Techn. 43, 249 [1968].