Primary Inducible Fluorescence in Secretory Granules of Tumor Cells Supravivally Stained by a Toluidine Blue Dye

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(Z. Naturforschg. 23 b, 969—975 [1968]; eingegangen am 20. Dezember 1967)

In a toluidine blue dye (British Drug Houses, batch no. 651880), we report a purple chromatographic fraction ($R_f=0.55$) with a spectral absorption peak at 622 m μ , which has the property to induce immediate bright yellow fluorescence (primary inducible fluorescence) in cytoplasmic granules of tumor cells. The cells are unfixed, supravital stained and examined by fluorescence microscopy. The mean number of fluorescent granules per tumor cell was 36, ranging from 19 to 64 (50 cells).

The effect of ultraviolet illumination on cells with fluorescent reaction is described, with reference to the shift of colour emission in the cytoplasmic bodies. These granules are Sudan Black and Oil Red positive, and become black after fixed cells are treated by osmium tetroxid. Actively growing cells accumulate basic dyes in lysosomes, and on this evidence, we base our belief that the granules of tumor cells with positive fluorescent reaction to this toluidine blue contain lipids, probably unsaturated fatty acids, are of secretory nature and might be derived from primary lysosomes.

The uptake of basic dyes by living lysosomes has now been reported by several authors. ZELENIN¹, who uses fluorescence microscopy to assess the distribution of fluorescent basic dyes in living cells, confirmed the lysosomal nature of the cytoplasmic bodies stained by acridine orange, by comparing electron microscope acid phosphatase preparations of isolated bodies with the structures seen on cells exposed to dye. DALCQ² used fertilized mammal eggs to study the formation of metachromatic granules stained by toluidine blue. These granules contain acid phosphatase and DALCQ² discussed the relation of this substance to lysosomes and phagosomes. Using acridine orange, neutral red, quinacrine and toluidine blue for vital and supravital staining of actively growing cells, we verified that these dyes had the same capacity to stain lysosomes and lysosome-derived bodies. When studying these staining properties under fluorescence microscopy, we observed bright yellow fluorescent cytoplasmic granules on a tumor cell preparation supravivally stained with toluidine blue. Toluidine blue is listed as a basic thiazin non-fluorescent dye (LILLIE³) and this finding prompted us to study the phenomena further.

It is the purpose of this paper to present the results of our studies on inducible fluorescence by toluidine blue in cytoplasmic granules of tumor cells.

Material and Methods

Staining Procedures

Supravital Staining: Precleaned microscope slides (76 × 26 mm) with fine ground edges were used. Two drops of 1% ethanol solution of toluidine blue were put on one surface of a slide and the solution was then evenly spread on both surfaces (with the other slide).

After the air had dried them, these slides were kept at room temperature in light and dust proof boxes. One drop of ascitic fluid obtained fresh from tumor-bearing mice (8 to 12 days after tumor transplantation) was put on a coverslip (24 × 50 mm), and it was then smeared on the toluidine blue prepared microscope slide. The number of cells is about 4×10^6 per mm³.

In Vivo Staining: Tumor-bearing mice were injected intraperitoneally with 1 ml of 1% saline dye solution. Tumor cells were collected 24 hours later. Cells from normal mice peritoneal cavity or from ascitic exsudate induced by intraperitoneal injection of glycogen served as a control. These studies were done with Sarcoma 37 tumor cells, ascitic form, while maintained by transplantation during 30 consecutive passages.

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¹ A. V. ZELENIN, Nature [London] 212, 425 [1966].

² A. M. DALCQ, in: A. V. S. REUCK and M. P. CAMERON (eds.), Lysosomes, J. and A. Churchill, London 1963.

³ R. D. LILLIE, Histopathologic Technic and Practical Histochemistry, 3th. ed., McGraw-Hill Book Comp., New York 1965.

Vital and supravital preparations were observed unfixed using the microscopic equipment used elsewhere (BASTOS et al.)⁴. Fluorescent micrographs are copies obtained from black and white film, Tri-X (Kodak), Isopan (Agfa) or HP3 (Ilford), after having been given a standard photographic exposure of 30 seconds.

Toluidine blue compounds (C.I. 52040), British Drug Houses, Poole, England, batch no. 651880, and no. 3034350; George T. Gurr, London, England, batch no. 18925; Chroma-Gesellschaft Schmidt Co., Stuttgart, Germany, batch no. 6105, and Grubler, Leipzig, batch unknown.

Chromatography: Shandon chambers for the column descendent method using the Whatman paper no. 3 MM. Five centimeter trace of a deposit of 0.1 ml of an aqueous solution of toluidine blue (10 mg/ml). Solvent mixture: tertamyl alcohol, p.a. 96 ml, acetic acid glacial, p.a. 26 ml, and bidistilled water, 40 ml.

Chromatographic separation time at room temperature was 16 hours. Each fraction on the chromatogram was eluted with 2 ml of ethyl alcohol and each solution was concentrated by evaporation under nitrogen.

Spectrophotometry: Unicam SP 800 Spectrophotometer was used to record the spectral absorption of the toluidine blue dyes in aliquots of 3 ml of 0.5% aqueous (bidistilled water) solution.

Results

Fig. 1-A represents the fluorescence microscopy appearance of Sarcoma 37 tumor cells supravitaly

stained with toluidine blue (B.D.H., batch 651880). Of the compounds tested, only this batch of toluidine blue had the property to induce immediate bright yellow fluorescence in cytoplasmic granules. This reaction is present in the cytoplasm of all tumor cells present in the smear. The fluorescent bodies evidenced by this batch of toluidine blue correspond, under light microscopic examination of fresh tumor cells, to high refractory bodies present on stained (Fig. 1-B) or unstained cells (Fig. 1-C). Not all the cytoplasmic granules stained by toluidine blue give the fluorescent reaction. Only bigger ones do. The mean number of fluorescent bodies counted per tumor cell was 36 with a range from 19 to 64, in 50 cells. In these preparations the extrusion of fluorescent bodies from tumor cells was commonly observed.

The fluorescent intensity of cytoplasmic granules decreases after 15 seconds of continuous ultraviolet light exposure, making color microphotographs difficult to record; in 30 seconds the color changes from bright yellow to blue and, following one minute of illumination, the fluorescence becomes diffuse. Using microscope slides prepared with dye solutions ranging from 1 per cent to 1/100000 (10 fold dilutions) for supravital staining tumor cells,

Fig. 1 A

⁴ A. L. BASTOS, J. F. B. M. NUNES, J. D. VIGÁRIO, A. M. TERRINHA, D. V. MARQUES, J. F. SILVA, and A. D. CORREIA, Rev. de Biologia [Lisbon], in press (1967).

Fig. 1 B

Fig. 1 C

Microscopic appearance of cytoplasmic granules with fluorescent reaction: (A) Supravitaly stained by Toluidine blue (batch 651880); (B) Same field under light microscopy; (C) Unstained and freshly obtained tumor cells. Note the imobility of cell membrane of Toluidine blue supravitaly stained cells (750:1).

demonstration of fluorescent granules was still possible on 1/10000 slides. On these preparations the color emission of the cytoplasmic bodies was blue.

The microscopic appearance of tumor cells, vital and supravital, stained by quinacrine or toluidine blue, is identical (Figs. 2-A and 2-B). Preliminary electromicroscopic studies (NUNES et al. manuscript in preparation) of Sarcoma 37 tumor cells with living lysosomes tagged by quinacrine showed (fixa-

tion by glutaraldehyde 2,5 per cent and post fixed in osmium tetroxide 1 per cent) detectable ultrastructural changes distributed mainly on the cytoplasm: increased number and pleomorphism of osmophilic bodies, autophagic vacuoles and/or cytolysosomes, mielin-like figures, well developed Golgi apparatus and in some cells possible mitochondria swelling (Fig. 3)*.

* Fig. 3 s. Tafel S. 970 a.

Fig. 2 A

Fig. 2 B

Freshly obtained ascitic sarcoma 37 cells intravitaly stained by (A) Quinacrine; (B) Toluidine blue (B.D.H., batch 651880). Fluorescent micrographs obtained 24 hours after an intraperitoneal injection of 1 per cent dye solution to a tumor bearing mice, (188:1).

Tables I and II summarize the results of both the absorption spectra and chromatographic studies of the toluidine blue compound tested. The spectral curves are similar to those reported by the Biological Stain Commission⁶ with a maximal absorp-

tion peak at 622 m μ . On the available commercial dyes we have found by chromatography as many as seven fractions, although a major blue component is present in all compounds which probably represents the true toluidine blue dye. Since the item no.

⁵ A. L. BASTOS, A. M. TERRINHA, J. D. VIGÁRIO, J. F. M. NUNES, and J. L. N. PETISCA, *Exp. Cell Res.* **42**, 84 [1966].

⁶ H. J. COHN, *Biological Stains*, 7th. ed., Williams and Wilkins, Baltimore 1961.

Table I. Absorption spectra of Toluidine blue solutions. Identification of curves. (1) British Drug Houses, batch 651880; (2) British Drug Houses, batch 3034350; (3) Chroma-Gesellschaft Schmid & Co., batch 6105; (4) George T. Gurr, batch 18925; (5) Grubler, batch unknown.

Toluidine blue dyes	Chromatographic spots	R_f
British Drug Houses Ltd. (batch 651880)	I	0,43
	II	0,48
	III	0,55
	IV	0,59
	V	0,88
	VI	0,93
George T. Gurr (batch 18925)	I	0,19
	II	0,23
	III	0,27
	IV	0,32
	V	0,55
	VI	0,69
	VII	0,77
Grubler (batch unknown)	I	0,06
	II	0,13
	III	0,24
	IV	0,49
	V	0,56
Chroma Gesellschaft Schmid & Co. (batch 6105)	I	0,18
	II	0,23
	III	0,27
	IV	0,33
	V	0,62
	VI	0,69
	VII	0,77

Table II. Results of chromatographic analysis of Toluidine blue dyes.

651880 (B.D.H.) has the property to induce bright fluorescence in cytoplasmic granules of tumor cells, each fraction on the chromatogram of this dye was eluted, concentrated to the desired level and alcoholic solutions prepared for supravital staining. We observed that only the III fraction (R_f 0.55) has the same fluorescent property as the total dye solutions. When another item no. 3034350 (B.D.H.) was tested, no fluorescence of cytoplasmic granules was seen. We verified by chromatography that the fraction (R_f 0.55) of item no. 651880 was not present on the chromatogram of item no. 3034350 (Fig. 4).

Fig. 4. Paper chromatographic analysis of British Drug Houses Toluidine blue dyes: (1) Batch 651880; (2) Batch 3034350. The component III (purple) confers the property of inducible fluorescence.

Discussion

In comparison to other cytochemical methods⁷, vital and supravital staining methods of lysosomes and lysosome like bodies with basic dyes are simple, reliable (i. e. supravital prepared slides keep staining properties for months), and give precise information about the distribution, number and size of these bodies on living cells. The supravital staining procedure by toluidine blue is now being used by us to evaluate clinical smears obtained from cancer suspect patients (cervical dysplasia, peripheral blood

leukemic and non-leukemic leucocytes, mamary and other body exudates). We had the opportunity to assess this toluidine blue stain on different cell systems in culture (mice embrionic cells, rat embrionic cells, rat embrionic liver cells, mice macrophags and KB cells) and from the experimental animal, nitrosamine induced hepatomas and mice spleen cells infected by Friend virus, and only tumor cells exhibit fluorescence of cytoplasmic granules, which are similar in form, size and distribution to the granules of Sarcoma 37 tumor cells. On preparations of mice spleen cells obtained from infected animals, intact stain granules were seen free on the intercellular space*. One of the advantages of the vital staining method for tumor cells, reported earlier⁸, is that the toluidine blue supravital staining of cell smears does not require any drug administration to the patient before the test. SMITH and CAMILLERI⁹ discussed the possibility of assessing cell malignancy in smears using acid phosphatase staining of lysosomes.

We believe, like others¹⁰, that the uptake of basic dyes by lysosomes of living cells (vital staining) is an active and energy requiring process. We observe

on cultured cells exposed to quinacrine that the pH of the medium (phenol red indicator) drops more rapidly on dye exposed cells than on control cells. The acid output is an indication of an increased metabolic activity of quinacrine treated cells, which are living, viable while lysosomes are tagged with quinacrine.

In exponential growing cells (mouse embryonic cells, sheep embryonic cells or a cell line of monkey kidney cells) incubated at 37 °C for 24 hours in a culture medium containing quinacrine at concentrations of 10^{-7} and 10^{-6} g/ml and examined in living state by fluorescence microscopy we detected bright yellow fluorescence in lysosomes of cells of the cultures. Interphase or mitotic nuclei are unstained (Fig. 5). On quinacrine exposed culture cells we found by autoradiography that 100 per cent of the interphase nuclei are labeled by tritiated uridine, 30 to 40 per cent are tagged by tritiated thymidine. Subsequent culture passages of dye exposed cells have always been successful. Autoradiographic kinetic studies of Sarcoma 37 tumor cells showed a cell growth fraction over 90 per cent, 25 per cent of the cells are in S compartment, the fraction of

Fig. 5. Mice embryonic cells after growing for 24 hours on a medium containing quinacrine (10^{-6} g/ml). Bright yellow staining is only detected in lysosomes. Nuclei do not stain with quinacrine, unless the cells are exposed to higher dye concentration or damaged, (750:1).

⁷ P. B. GAHAN, *Int. Rev. Cytol.* **21**, 1 [1967].

⁸ A. L. BASTOS, *Acta Cytol.* **10**, 18 [1966].

⁹ C. J. SMITH and G. E. CAMILLERI, *Nature [London]* **201**, 512 [1964].

¹⁰ E. ROBBINS, P. I. MARCUS, and N. K. GONATAS, *J. Cell Biol.* **21**, 49 [1964].

tritiated thymidine labeled mitosis reaches 100 per cent 6–7 hours after the pulse labelling and the mean generation time calculated for this ascitic tumor was 17 hours¹¹. We know therefore that this cell population is entirely proliferative and all tumor cells exhibit fluorescence of cytoplasmic granules when supravitaly stained with toluidine blue. The significance of the fluorescent reaction might indicate a selective binding for a lysosome component, a particular functional state of these bodies associated to cell physiology, or both. The control of lysosome enzyme activation has been associated with the regulation of the cell cycle^{12–14}.

The extrusion of fluorescent bodies from tumor cells, besides possible implications in cell invasion due to the hydrolytic nature of the granules, supports the concept associating primary lysosomes to secretory granules. The fluorescent reaction of the granules to toluidine blue might indicate the secretory nature since, under light microscopic observation, the smaller granules stained by toluidine blue are not fluorescent and might represent primary lysosomes. Lipids and their oxidation products are fluorescent¹⁵. KOENIG¹⁶ related autofluorescence of lysosomes to lipid autooxidation. The refractory bodies of tumor cells which give the fluorescent reaction are sudanophilic (0.3 per cent solution of Sudan Black in ethanol) and oil red positive (0.5 per cent solution of oil red O in ethanol), and are black when tumor cells are fixed by glutaraldehyde 2.5 per cent and post fixed in osmium tetroxide 1 per cent. The reduction of osmium tetroxide indicates usually the presence of unsaturated fatty acids and fatty acids⁴. The color changes of the fluorescent granules (yellow to blue) after cell exposure to ultraviolet light might result in oxidation

either of the lysosome phospholipid membrane and/or the glycolipoproteic matrix (PAS positive substances). One physicochemical basis for the color shifting of the fluorescent granules under ultraviolet illumination is a decrease dye (cationic) binding to cell component due to oxidation. When tumor cells are supravitaly stained on slides prepared from dye solutions at concentrations of 1/10000 the fluorescent color emission is blue.

During a systematic search among thiazin dyes (azure I, azure II, azure A, methylene blue, toluidine blue and thionin) we found that all these dyes have the property to induce fluorescence in cytoplasmic granules (secondary inducible fluorescence), but only after a period of tumor cell exposure to ultraviolet light. We are planning to explore by microfluorometric methods the fluorescent structure latency of the granules to thiazin dyes and ultraviolet light exposure after obtaining tumor cells in various conditions.

At molecular level the phenomenon of inducible fluorescence is assumed to be a process of photonic energy transfer from the sensitizer (dye) to a cell component, the resulting spectral fluorescence emission being specific of the second compound. These considerations might help to identify the chemical structure of tumor cell granules responsible for the fluorescent reactions which are at present unknown at molecular level.

Studies carried out by one of us (A. L. B.) in the "Max-Planck Institut für Virusforschung, Abt. für physikalische Biologie (Direktor: Prof. Dr. H. FRIEDRICH-FREKSA)", Tübingen, Germany. We want to thank Eng. Agr. JOSÉ CONTREIRAS, Department of Plant Physiology, Estação Agronômica Nacional, Oeiras, Portugal, for the use of Unican SP 800. This work was supported by a Calouste Gulbenkian Foundation Grant.

¹¹ L. L. FIGUEIREDO, M. D. Thesis, Lisbon University 1967.

¹² A. C. ALLISON and L. MALLUCCI, *Lancet* **2**, 1371 [1964].

¹³ R. HIRSCHHORN, J. M. KAPLAN, A. F. GOLDBERG, K. HIRSCHHORN, and G. WEISSMANN, *Science* [Washington] **147**, 55 [1965].

¹⁴ A. L. BASTOS, J. F. B. M. NUNES, J. D. VIGÁRIO, J. L. N. PETISCA, and A. M. TERRINHA, *Z. Krebsforsch.* **69**, 1 [1967].

¹⁵ T. BARKA and P. J. ANDERSON, *Histochemistry*, 1st. ed., Harper and Row Publ., New York 1963.

¹⁶ H. KOENIG, *J. Histochem. Cytochem.* [Baltimore] **11**, 120 [1963].

Fig. 3. Electron-micrograph of four Sarcoma 37 tumor cells. L — Lysosomes and lysosome-derived bodies (autophagic vacuoles and/or phagosomes). M — Myelin like figures (phospholipid), Mt — mitochondria. On dye exposed cells the euchromatic, heterochromatic and nucleolar regions of

the nucleus are identical to those of untreated tumor cells. These tumor cells were stained by uranyl acetate and were obtained from tumor bearing mice like the cells shown on Fig. 2 A. Enlargement 21000.