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Microdensitometrical studies on tumour-induced programmed cell death of peripheral blood tumour-infiltrating lymphocytes (TIL) in cancer patients: possible applications for early tumour diagnosis

*E. Zvetkova, G. Kostov**

Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, Sofia
**Oncological Hospital, Veliko Tarnovo*

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Automated (scanning) cytospectrophotometric analysis of the nuclear DNP-content and distribution, based on the highly sensitive microdensitometric criteria as nuclear area, intensity of staining (light transmission), optical density (light absorption), DNP distribution in the histograms of individual nuclei etc., has great information capability for the purposes of automatic selection of the normal and abnormal peripheral blood cells, including circulating TIL (tumour-infiltrating lymphocytes) in cancer patients. Possible applications of this method for early diagnosis of tumours has been proposed.

Key words: Tumour infiltrating lymphocytes (TIL), programmed cell death (apoptosis), nuclear DNP condensation and distribution, microdensitometrical/cytospectrophotometrical analysis, peripheral blood smears, cancer patients, early tumour diagnosis.

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Introduction

We have previously reported [1 - 7] that some cytochemical differences with regard to the quantity and distribution of cell nucleoproteins (RNP and DNP) in different lymphocyte populations from the peripheral blood of patients with carcinomas, could be possible criteria for determining the course and prognosis of neoplastic disease, as well as for individualization of patients' treatment.

The aim of this study was a development of analytical microdensitometrical system for the purposes of diagnostic selection of the so-called tumour-infiltrating lymphocytes (TIL) circulating in the peripheral blood of cancer patients and automatically registered by deviations in their individual nuclear histograms as well as in the summarized histograms of TIL cell populations.

Material and methods

The study was carried out on human lymphocytes from peripheral blood smears of 10 healthy individuals (used as controls) and from 10 patients with neoplasms (5 men with lung cancer and 5 women with breast tumours).

Smear preparations were stained by the methylene blue-fast green staining method of E. Zvetkova and I. Zvetkov [8]. Two staining variants of the method were employed: 1.the combination of methylene blue-fast green for the simultaneous detection of nuclear deoxyribonucleoproteins (DNP) and/or of ribonucleoproteins (RNP) in the cell cytoplasm and nucleoli; 2. the staining variant with cold acid (5N HCl) hydrolysis which contributes to the acid extraction of the cellular ribonucleic acids (RNAs), the nuclear DNP being selectively stained by methylene blue, according to the degree of chromatin dispersion and condensation (in dependence on the state of the nuclear chromatin activation/inactivation).

Morphometrical/cytospectrophotometrical studies [9] were carried out after the staining according to the variant of acid (5N HCl) hydrolysis. Measurements were accomplished by means of a scanning microscope-photometer "Opton" at a visible light wave length of 560 nm, a sound of 0.5 μm and a step of the movable table 0.5 μm. The quantitative data were presented in individual cell histograms registering DNP condensation and distribution in lymphociyte nuclei, as well as in the summarized histograms of lymphoid (TIL) populations.

For transmission electronmicroscopic (TEM) examination the freshly collected, cytocentrifuged (at 600 - 800 g) peripheral blood lymphocytes, were embedded in gel drops and processed for electron microscopy after in situ fixation with phosphate-buffered 3% glutaraldehyde and posfixation in 1% osmium (OsO_4) solution. Semithin sections of 1 μm were stained with Azur B and examined by light microscope "Opton". Ultrathin sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscope "Opton" EM-10A.

Results and discussion

The results obtained display possibilities of the staining method applied [8] for cytological diagnostic studies (Fig.1 and 4) as well as for automatic cytophotometrical selection of functionally different peripheral blood lymphocytes in the blood smears of healthy persons and/or of cancer patients (Fig. 2, 3).

Explanation of quantitative results in these cases could be done on the basis of our previous cytochemical data [1 — 7] on the abnormalities in cytochemical characteristics and especially in quantity and distribution of nucleoproteins (RNP and DNP) in the population of small and medium-sized (7 - 8 μm diameter, with little cytoplasm) tumour-infiltrating lymphocytes (TIL), circulating in the peripheral blood (PB TIL) of cancer patients and tumour-bearing animals:

- Quantitative alteration/reduction in the cytoplasmic RNP-contents (RNP-granules) of the small and medium-sized TIL from the peripheral blood of cancer patients and tumour-bearing animals are well presented on Fig. 1 — *a, b, d — g* and Fig. 4 — *a — c*. The same T-lymphocyte subpopulation begins to secrete its cytoplasmic RNP-containing granules [5] upon lymphocyte (killer cell) - tumour (target) cell conjugation and adhesion (Fig. 1 — *a, b*; Fig. 4 — *a*). On the delivery of TIL secretory granules to the tumour cell membrane and vice-versa, the cytoplasmic RNP, occurring in the cytoplasms of both cells — tumour and lymphocyte, remain unstained only in places of cell to cell contacts (Fig. 1 — *b*). In some cases RNP-containing cytoplasmic clasmatoses could form "bridges" between killer and target cells (Fig. 4 — *a*). These changes in

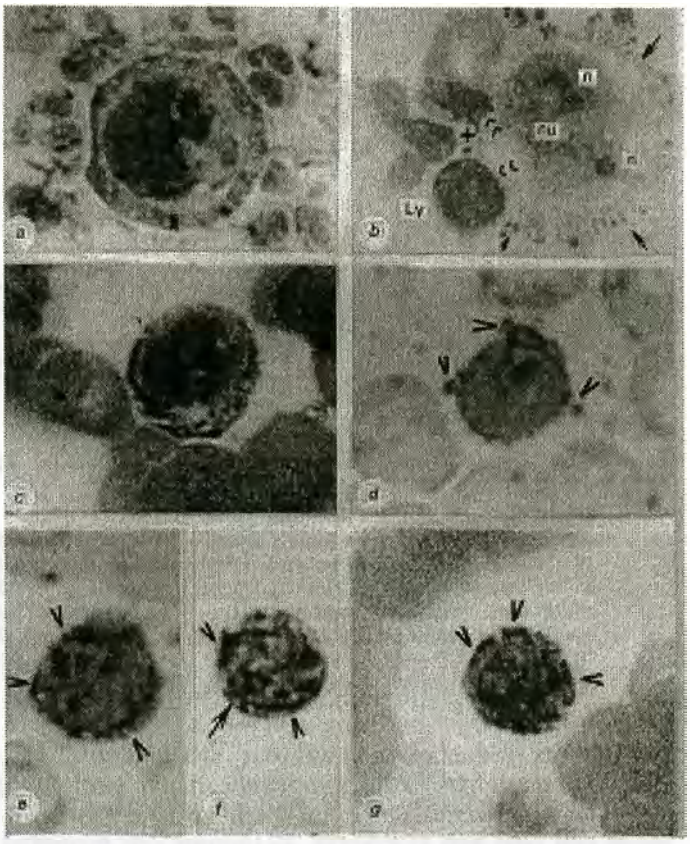

Fig. 1. Cancer cells (big ones, with dispersed chromatin in nuclei (n) and active nucleoli (nu) and blood cells (granulocytes and lymphocytes (Ly) interrelationships: *a, b* — cytoplasmic RNP (<<) are unstained in both - cancer cell and TIL (tumour infiltrating lymphocyte) only in places of cell to cell contacts; *c* — small PB T-lymphocyte from healthy person, with normal quantity and distribution of cytoplasmic RNP; *d, e, g* — small tumor-infiltrating lymphocytes from the peripheral blood (PB TIL) of cancer patients, with reduced (soon by microclasmatoses (1g) >) and unevenly distributed cytoplasmic RNP (>); *f* — unevenly distributed hypercondensed DNP in the nucleus of PB TIL of cancer patient - one could see voluminous perinuclear/subcaryolemal heterochromatin clumps (>) as well as multiple dot-like structures of condensed nuclear DNP in the same nucleus (→). × 600, Immersion. The staining method of E. Zvetkova and I. Zvetkov [8]: *a–e, g* — the staining variant without cold acid hydrolysis; *f* - staining after 5N HCl hydrolysis; Microscope "Opton"

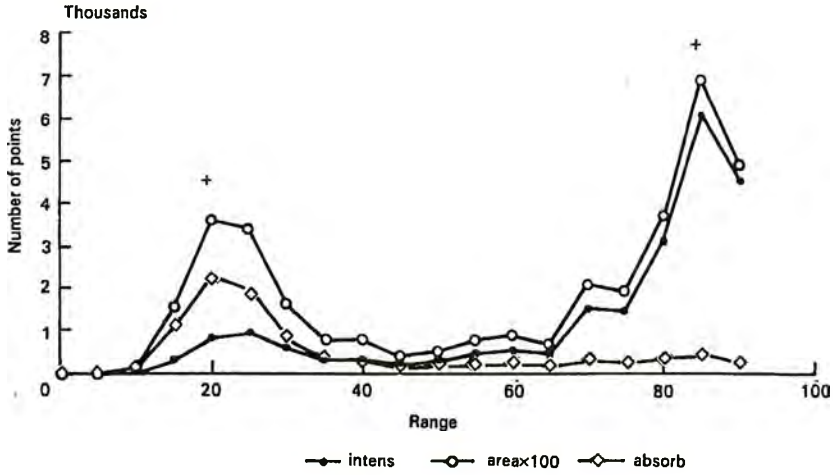

Fig. 2. Histogram of PB T-lympocyte from healthy person
One could see evenly distributed nuclear DNP in the nuclear area, forming mainly two maximums: first, smaller one - at the range 18-25, in the zone of condensed chromatin (heterochromatin) and the second - at the range 70-80, in the zone of euchromatin (decondensed nuclear chromatin). The intensity of nuclear DNP staining (intens) and the degree of light absorption (absorb) have been also registered. The staining variant of the methylene blue-fast green method, applied after cold acid hydrolysis [8]; Immersion, Cytophotometer "Opton"

the quantity (reduction) and unevenly distribution of TIL cytoplasmic RNP [1-3] are first signs or signals of TIL programmed cell death (apoptosis).

The precise molecular mechanisms that represent lymphocyte-mediated tumour cell lysis and vice-versa — tumour cell mediated lymphocyte programmed cell death, are unclear: a possible influence of citolytic proteins — perforin, integrins and several proteases or other biologically active substances, involving changes in the cellular plasma membrane permeability and a secondary cytoplasmic and nuclear damage, has been discussed [5, 10].

The early appearence of PB TIL with reduced and unevenly distributed cytoplasmic RNP in the peripheral blood of cancer patients, such with precanceroses and tumour-bearing animals, could be successfully used as a diagnostic sign of neoplastic (preneoplastic) disease,reflecting first biological events of the fundamental effector mechanisms of the immune system for the elimination of tumour cells [1 — 5]. Unfortunately these possibilities are largely unexplored in diagnostics [11, 12], but it will be interesting to observe the up-to-date development of this area of research in relationship to quantitative data on changes in the nuclear chromatin DNP-condensation and distribution of the same T-lymphocyte (PB TIL) subpopulation:

- Cancer patients' PB TIL are small and medium-sized T-cells with more condensed and unevenly distributed deoxiribonucleoproteins (DNP) in the nuclear chromatin, forming large irregular clumps of heterochromatin, localized preliminarily in the nuclear periphery [perinuclear/subcaryolemal zones — Fig. 1 — *f*; Fig. 4 — *d*; (see also 1 - 7)]. In the central parts of these nuclei one could also see a presence of multiple, very small in size, condensed DNP-clusters, alternating with dis-

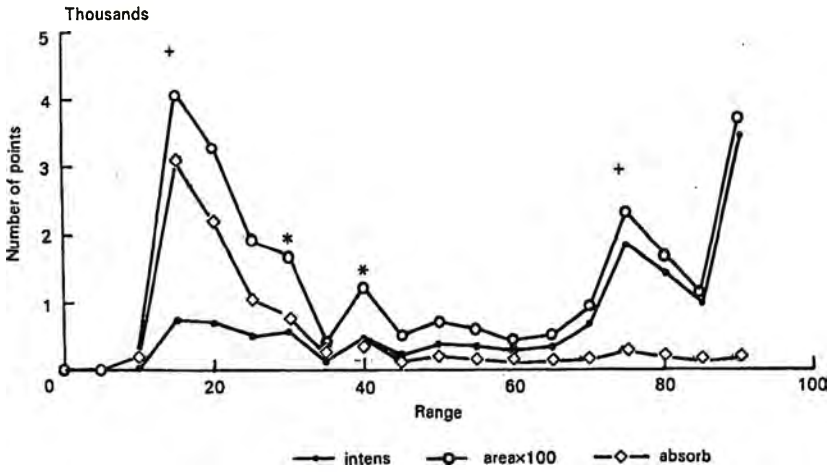

Fig. 3. Histogram of PB TIL of cancer patient with more condensed and unevenly distributed DNP in the nuclear chromatin (in comparison to the histogram of T-lymphocyte from healthy person, presented on Fig. 2)
In the zone of condensed nuclear chromatin a big peak (+) is situated in the lower ranges — at the range 15-20, without forming of plateau as in healthy person (see Fig. 2); the peak of dispersed nuclear chromatin (euchromatin) at the range 70 is smaller (+), than in healths. One or more additional peaks (*) are formed in the zone of heterochromatin in the lower ranges (30-40), probably due to uneven distribution ob abnormal DNP - localized subcaryolemally and in the nuclear dots. The staining varint of the method [8] - after 5N HCl hydrolysis; Immersion, Cytophotometer "Opton"

persed small euchromatin areas, which contribute to the characteristic micromacular nuclear appearance (Fig. 1 — *f*; Fig. 4 — *d*). A mainly subkaryolemal distribution of the more dense, voluminous and irregularly condensed DNP in the PB TIL nuclear chromatin occurring as chromatin perinuclear thickenings (clumps, blebs), contributes to the irregularly formed, undulated or lobulated nuclear periphery (nuclear microhypersegmentation — Fig. 1 —*f;* Fig. 4—d). In the irregularly formed PB TIL's nuclear periphery the inner and the outer sheets of nuclear membranes were soon damaged: in some cases the exstrusions of nuclear material in the cytoplasm are cleary visible in the electronograms of cancer patients' PB TIL (Fig. 4 — *d*).

Similar modifications of the surface and the form of nuclei, as well as ultrastructural changes in the nuclear membrane sheets were described by us also in the granulocytes (neutrophils, eosinophils and basophils) from the peripheral blood of cancer patients and such with precancerous states [1 - 3; 6]. However, it has been demonstrated by us [1 - 3] and by other authors [13 - 15] that not only mononuclear cells, but also granulocytes are involved in the response against the presence of the malignant tumour cells in the body (Fig. 1 — *a*). In the literature [6, 16 - 21] heterogeneous staining patterns and modifications in the amount and distribution of some nuclear envelope-associated components and nuclear envelope-limited chromatin sheets were analysed ultrastructurally, cytospectrophotometrically and by immunofluorescent and immunoblotting procedures, called "nuclear blebs", "nuclear projections", "nuclear appendages" and "nuclear pockets" in a variety of lymphomas, leukaemias etc. In some cases controllable systems for examining the mechanisms

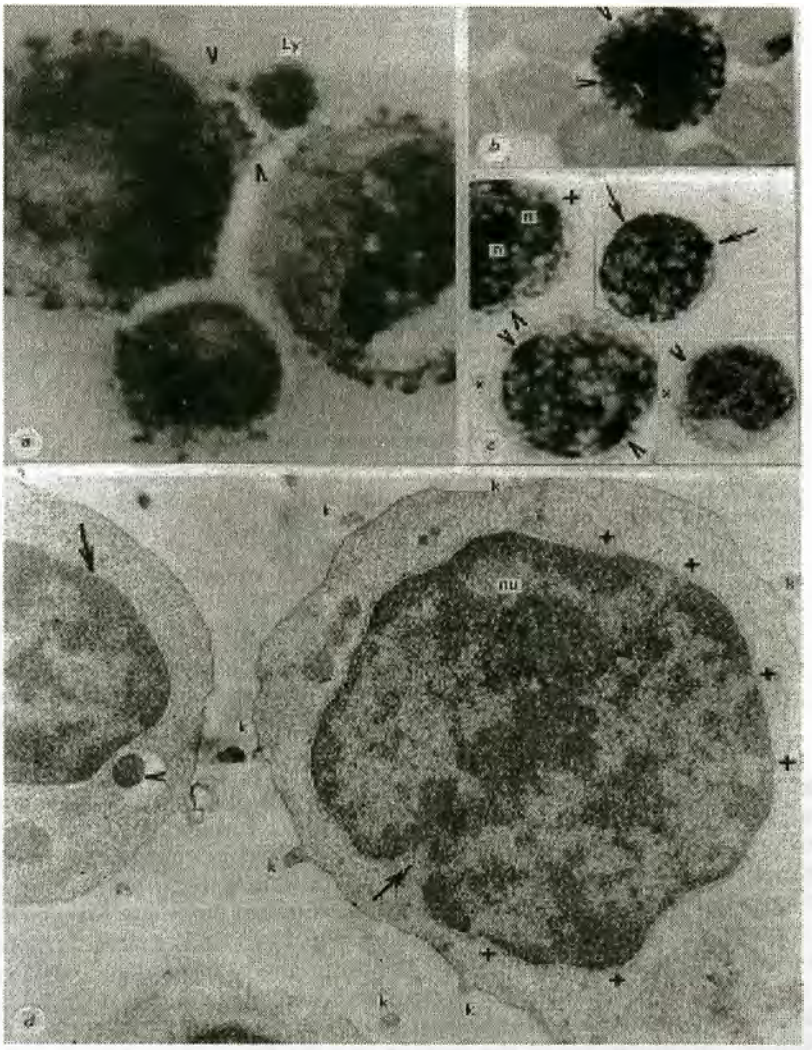

Fig. 4. TIL of cancer patients
a - Cancer cell-TIL interrelationships through RNP-containing intercellular bridges and/or clasmatoses (>); one could see reduced cytoplasmic RNP-granules in the lymphocyte (Ly); *b* - heterochromatization of nuclear chromatin and reduced in number cytoplasmic RNP (>) in the PB TIL of cancer patients; *c* - PB TILs from cancer patients showing reduced in number and unevenly distributed cytoplasmic RNP (>), changes in the shape of nuclei (→) reduced number of active nucleoli (+ - *n*) and a great number of resting (small, multiple, inactive - in some cases heterochromatized) nucleoli; *d* - electronogram of PB TILs from cancer-patient, with ribosomes-containing cytoplasmic clasmatoses (*k*), Changes in the nuclear membrane sheets (+), as well as in the nuclear contour (shape) [→] with extrusions of nuclear chromatin material in some cells (>); one could see irregular and unevenly distributed heterochromatin clumps at the nuclear periphery, as well as dot-like heterochromatin structures in the inner part of the nucleus; nucleolus (NU) is not active, forming the so-called resting nucleoli and/or the first nuclear dots. Opton electron microscope, × 6000

of nuclear lobulation and perturbation of the nuclear envelope - chromatin interactions were demonstrated [22].

Examined microdensitometrically/cytospectrophotometrically, the observed particularities of the contents and the distribution of DNP in the nuclei of PB TIL from the peripheral blood of cancer and pre-cancer patients may serve as diagnostic criteria in studying the course and the stage of the neoplastic process. In this regard cytochemical (qualitative) data are more limited in scope as compared to the guantitative — microdensitometrical (cytospectrophotometrical) parameters in the historams of individual lymphocyte nuclei recorded (Fig. 2, Fig. 3). Individual nuclear histograms have been obtained by means of direct measurements (on PB TIL from blood smears): of the nuclear area, the optical density, the intensity of staining and space distribution of DNP in the nuclear chromatin. In histograms some important quantitative, malignancy-associated changes in nuclei of PB TIL of tumour-bearing hosts, compared to healthy persons, were obtained (Fig. 2, Fig. 3):

- Prevalent condensed DNP with uneven distribution in the nuclear chromatin of cancer partients' PB TILs, forming specific peaks — one big (principial) and one-two additional, in the zones of condensed chromatin / heterochromatin of the histograms (Fig. 2), in comparison to the even nuclear chromatin distribution in normal PB TILs, where one or two peaks of euchromatin are very well expressed (Fig. 3). These specific microdensitometrical nuclear chromatin characteristics of PB TILs in cancer patients could be related to the signs of its malignancy-induced programmed cell death [5 - 7]: condensation of nuclear chromatin, nuclear membrane blebbing, nuclear chromatin fragmentation, and finally — the formation of apoptotic bodies (see reviews: 23, 24, 27, 28). The events could also be related to the formation of specific nuclear domains, known as "nuclear dots" (NDs) and discovered in the contents of neoplastic and leukaemic cell transformation; such structures containing specific proteins — in some cases covalently linked to the proteins of the nuclear pore complex and/or associated with viral DNA replication domains as well as with the presence of abnormal oncoprotein products [25 — 27].

- Deviations in the nuclear contour in the lymphocytes from patients with neoplasia, probably related not only to the subcaryolemal chromatin changes, but also to disruptions in the nuclear membrane sheets [22].

The results obtained could encourage further investigations regarding our concept of malignancy [1-3] as a "systemic nuclear disease" and about probable involvement and significance of the nuclear membrane in these relationships. Although these hypothesis should be examined by further experiments, it is also important to elucidate the in vivo cell death-inducing activity of the PB TIL resting nucleoli [29] some of which — with highly suppressed nucleolar RNA synthesis, could be transformed in the first nuclear dots/bodies — containing biochemically abnormal DNA and oncoproteins.

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