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To the nucleolar bodies (nucleoli) in cells of the lymphocytic lineage in patients suffering from B – chronic lymphocytic leukemia

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The present study was undertaken to provide more information on nucleoli in lymphocytes of B - chronic lymphocytic leukemia. The computer assisted nucleolar and cytoplasmic RNA image densitometry, reflecting the nucleolar and cytoplasmic RNA concentration at the single cell level, demonstrated a remarkable stability during the differentiation and maturation of B- lymphocytes. In contrast, as it was expected, the nucleolar diameter during the lymphocytic development markedly decreased. Thus the nucleolar RNA content of leukemic B-lymphocytes was apparently related to the nucleolar size. In both immature and mature lymphocytes, the cytostatic treatment increased the incidence of micronucleoli, which represent the „inactive" type of nucleoli. However, the decreased values of the nucleolar diameter were statistically significant only in mature lymphocytes of treated patients. On the other hand, despite such observation, it must be mentioned that „large active" and „ring shaped resting" nucleoli were still present in immature and mature lymphocytes after the cytostatic therapy and such cells might represent a potential pool of proliferating cells. As it is generally accepted „large active nucleoli" with multiple fibrillar centers are known to be characteristic for proliferating cells. „Ring shaped resting nucleoli" are present in sleeping cells, which may be stimulated to return to the cell cycle and to proliferate again. In addition, the nucleolar RNA distribution also indicated that Gumprecht ghosts mostly originated from mature lymphocytes. Increased ratio of the nucleolar to cytoplasmic RNA density in Gumprecht ghosts or apoptotic cells and apoptotic bodies of the lymphocytic origin was related to the decreased cytoplasmic RNA concentration. The increased nucleolar size together with the markedly decreased cytoplasmic RNA concentration characteristic for Gumprecht ghosts just reflected the spreading of lymphocytes during smear preparations. In apoptotic cells or bodies of the lymphocytic origin, the "frozen" nucleolar RNA concentration accompanied by a reduced RNA concentration in the cytoplasm exhibited a remarkable similarity to the apoptotic process induced in vitro by the cytostatic treatment.

Keywords: B-chronic lymphocytic leukemia; lymphocytes; nucleolar classes; size; nucleolar RNA image density -concentration

It is generally accepted that nucleoli are multifunctional cell organelles that participate in cell proliferation, differentiation, maturation, aging and cell death [1–6]. Nucleoli also represent sites of the ribosomal RNA transcription and assembly of ribosomal particles that migrate to the cytoplasm and represent a substantial part of the cell proteosyntetic machinery [7, 8]. It is also known that the basophilic properties of nucleoli and cytoplasm due to the presence of RNA decrease during the differentiation and maturation of blood cells [9–12]. On the other hand, quantitative information on the RNA density reflecting the RNA concentration in nucleoli and cytoplasm in during the differentiation and maturation of blood cells is very limited. Moreover, earlier quantitative cytometric studies suggested that nucleoli in maturing leukemic lymphocytes disappeared [10]. Previous studies using more sensitive method for RNA detection reported a „symmetric" decrease of the nucleolar and cytoplasmic RNA concentration during differentiation and maturation of erythroblasts [13]. In contrary, in differentiating and maturing granulocytes, the RNA concentration decreased „asymmetrically" since the cytoplasmic RNA concentration in advanced stages of the granulocytic development decreased earlier than that of nucleoli [14].

The present study was undertaken to provide more information on the RNA image density, i.e. RNA concentration in nucleoli and cytoplasm of immature and mature B- lymphocytes using the computer assisted image densitometry *in situ* at the single cell level. For this study, RNA in nucleoli and cytoplasm was visualized in unfixed peripheral blood smears by a simple and sensitive cytochemical procedure that facilitated seeing clearly not only large but also very small nucleolar bodies – micronucleoli – with characteristic RNA distribution [5, 15–17]. The peripheral blood of patients suffering from chronic lymphocytic leukemia represented a convenient

model for RNA density measurements of individual cells because of a sufficient number of both early and advanced stages of the lymphocytic B lineage in the peripheral blood. In addition, the morphology of differentiating and maturing lymphocytes is well known and each stage is characterized by the nucleolar size as well as nucleolar RNA distribution that reflect the incidence of various nucleolar types [e.g. 1, 5, 11, 12, 18–21], but mostly without quantitative data on the nucleolar and cytoplasmic RNA concentration [10, 11, 12, 21, 22]. It should be also mentioned that any information on quantitative changes of the RNA concentration in nucleoli and cytoplasm of various differentiation stages of lymphocytes at the single cell level might be a very useful tool for understanding studied blood disorders

Materials and methods

Nucleoli, i.e. nucleolar bodies without the perinucleolar chromatin, were studied in immature and mature lymphocytes in 20 patients suffering from B – chronic lymphocytic leukemia without any respect of the clinical state and therapy. 10 patients were untreated and 10 patients were treated with current cytostatic therapy (Chlorambucil, Cyclophosphamide, Fludarabine, Mabthera, Mabcampath [23] at the time of taking peripheral blood samples for the present study. The median of the lymphocytic counts in studied patients was larger than 35^9/L and provided a satisfactory number of these cells for the present study. For control of the size measurements, the nucleolar diameter was also determined in mature lymphocytes in peripheral blood smears of 6 healthy blood donors. The peripheral blood samples of studied patients and blood donors were taken for diagnostic purposes and the ethics committee of the Institute approved the protocols for the present study.

RNA of nucleolar bodies and cytoplasm was visualized without preceding fixation in peripheral blood smears by a simple and sensitive method using methylene blue buffered with McIlvain´s buffer [15, 16]. The pH 5.3 and citric acid of the buffer prevented the loss of RNA from unfixed cells in dry smears that were not older than 24 hours. For control of the size measurements, the nucleolar diameter was also determined in mature lymphocytes in peripheral blood smears of 6 healthy blood donors. The used method also facilitated to distinguish main nucleolar types and determine their incidence in both immature and mature lymphocytes of all studied patients.

Micrographs were captured with a Camedia digital photo camera C.4040 ZOOM (Olympus, Japan) placed on Jenalumar microscope (Zeiss, Germany). The double adapter on the microscope increased the magnification of captured images on the computer screen. Increased contrast of stained nucleolar bodies by image processing facilitated easy measurements of the nucleolar diameter using Quick Photoprogram (Olympus, Japan) to provide basic information on the nucleolar size.

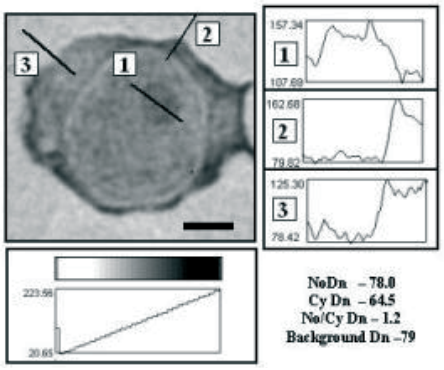

Figure 1. Nucleolar (1) and cytoplasmic RNA image density (2,3) measured in a lymphoblast. The numbers within density graphs are measured values. The calculated arbitrary density units for nucleoli (NoDn) or cytoplasm (CyDn) and the No/CyDn ratio are in the lower right corner of the Figure. Black bars in the micrograph indicate the measured lines. The density graphs are marked according to measured lines in the micrograph. The density scale is below the micrograph. The thick black bar in this and following Figures represents 2µm.

The nucleolar and cytoplasmic RNA image density was measured after the conversion of captured colored images (predominantly blue signals) to gray scale using the red channel (NIH Image Program, Scion for Windows, Scion Corp., USA). The RNA image density reflecting the RNA concentration in nucleoli and cytoplasm was expressed in arbitrary density units calculated by subtracting the mean background density surrounding each measured cell from measured nucleolar or cytoplasmic densities [13]. The background and cytoplasmic densities were measured in two locations, which exhibited the lowest and highest positivity (Fig. 1). Such calculations and standardization of arbitrary density units facilitated the comparison of results in defined regions, i.e. monolayers of peripheral blood smears [11]), which usually exhibited different artificial densities due to smear preparations from various patients. This approach decreased artificial measurements and thus provided better results than the background adjusted to zero, which depended on the investigator.

The results of measurements such as mean, standard deviation and significance were evaluated using "Primer of Biostatistic Program, version 1" developed by S.A. Glantz (McGraw-Hill, Canada, 1968). Mean of nucleolar to cytoplasmic RNA image density ratio for studied cells was calculated from mean values of nucleolar and cytoplasmic RNA image densities (nucleolar RNA image density divided by cytoplasmic RNA image density).

Results

The quantitative data are presented in the Table 1 and 2. The term nucleoli represents nucleolar bodies stained for RNA without the perinucleolar chromatin.

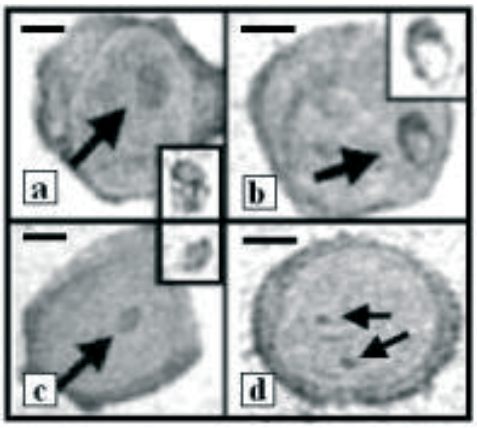

Figure 2. Immature lymphocytes. (a) A large nucleolus (arrow) with multiple small regions corresponding to fibrillar centers and trabecular structures (see the insert). (b) A large ring shaped nucleolus (arrow) with a very thin RNA containing peripheral ring and cap (see the insert). Such cap formation was observed after cytostatic treatment blocking the RNA transcription (ref. Busch and Smetana, 1970). (c) A ring shaped nucleolus (arrow) with a distinct opening of the peripheral ring (see the insert). (d) Micronucleoli (arrows).

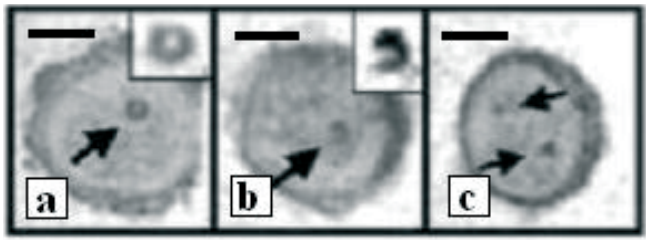

Figure 3. Mature lymphocytes. (a) A ring shaped nucleolus (arrow) with a distinct peripheral RNA containing ring (see the insert). (b) A ring shaped nucleolus (arrow) with a wide opening of the peripheral ring (see the insert). Such phenomenon is known to be in aging or altered cells. (c) Micronucleoli (arrows).

The incidence of main nucleolar types and values of the nucleolar coefficient (Table 1)

Immature lymphocytes mostly contained single large nucleoli with less or more uniform distribution of RNA containing structures (Table 1). Such nucleoli are known to be convenient markers of proliferating cells [5, 22] and contain multiple small-unstained areas corresponding to AgNORs that represent nucleolar fibrillar centers (Fig. 2a, ref. 5). It is generally accepted that the periphery of these nucleolar components is involved in RNA transcription. [5]. The incidence of such nucleoli in immature lymphocytes decreased in patients treated with cytostatic therapy. However, the difference was not significant because of a large variability with variation coefficient that reached 48 to 54%. Similarly, the decreased incidence of ring shaped nucleoli with reversibly decreased RNA transcription [5] in immature lymphocytes of treated patients was not significant. However, the peripheral RNA containing ring was occasionally irregular and exhibited a distinct opening (Fig 2b, c). In contrary, micronucleoli in immature lymphocytes in these patients were significantly more frequent (Fig. 2d). The significantly increased incidence of micronucleoli and not-significant decrease of large nucleoli and ring shaped nucleoli indicated that micronucleoli originated from both these nucleolar types. It should be mentioned that micronucleoli reflect the cessation of the RNA transcription [5]. It should be also mentioned that in treated patients some large ring shaped nucleoli frequently exhibited an alteration that was described previously and resulted in transformation to micronucleoli (not shown). In addition, the values of the nucleolar coefficient (number of nucleoli per cell) of lymphoblasts in patients treated with cytostatics also increased.

Table 1. The incidence of main nucleolar types and nucleolar coefficient (number of nucleoli per cell) [a, b]

Cells	Therapy	large	ring shaped nucleoli	micro	No coeff
Lybl	0	**62.7** ± 34.0	**33.5** ± 33.7	**3.8** ± 6.2	**1.07** ± 0.13
	+	**47.1** ± 22.9	**20.5** ± 12.2	**21.5** ± 17.2#	**1.37** ± 0.38#
Lycyt	0	**6.1** ± 1.3*	**58.8** ± 15.0*	**35.6** ± 15.0*	**1.23** ± 0.08*
	+	**1.4** ± 0.3*#	**44.0** ± 18.0*	**54.6** ± 12.0*#	**1.31** ± 0.08#

Legend
a – based on 10 untreated and 10 treated patients with cytostatic therapy
b – Mean and standard deviation
* – significantly different from lymphoblasts using t-test ($p<0.04$)
# – significantly different from patients untreated with cytostatic therapy using t-test ($p<0.03$)
Lybl – lymphoblasts and immature lymphocytres, Lycyt – mature lymphocytes

Mature lymphocytes of untreated patients mostly possessed „sleeping" ring shaped nucleoli (Fig. 3a). „Inactive" micronucleoli (Fig. 3c) were less frequent and „active" large nucleoli with more uniform distribution of RNA were noted exceptionally. In treated patients, the incidence of micronucleoli was markedly increased similarly as the number of nucleoli expressed by values of the nucleolar coefficient. At this occasion it should be mentioned that some of ring shaped nucleoli exhibited a large opening of the peripheral RNA containing ring (Fig 3b)

Nucleolar diameter, the nucleolar and cytoplasmic RNA image density (Table 2)

As it was expected, the nucleolar diameter was markedly smaller in mature than in immature lymphocytes in both untreated and treated patients with cytostatic therapy. However, the small differences of the nucleolar diameter between immature lymphocytes in untreated and treated patients were

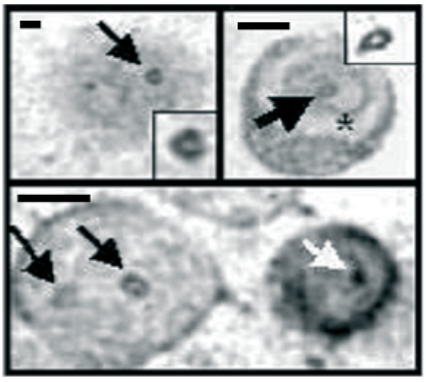

Figure 4. A Gumprecht ghost, apoptotic cell and body originating from lymphocytes. (a) A Gumprecht ghost with a micronucleolus and a ring shaped nucleolus (large arrow and insert). Note that the smeared and altered cytoplasm almost lost the positivity for RNA. (b) An apoptotic lymphocyte with a ring shaped nucleolus (arrow and insert). The unstained but distinct condensed chromatin at the nuclear membrane – asterisk. (c) A mature lymphocyte with two nucleoli (black arrows) and a smaller apoptotic body with a micronucleolus (white arrow).

not statistically significant. It seems to be due to the large variability of measurements in treated patients (the variation coefficient reached 15%, see also the large standard deviation). On the other hand, the small decrease of the nucleolar diameter in lymphocytes of treated patients was significant since the standard deviation was small and the variation coefficient was smaller than 10%. It seems to be interesting that the nucleolar diameter in lymphocytes of leukemic patients treated with the cytostatic therapy (1.04 ± 0.07 μm) was very similar to that in healthy blood donors (1.05 ± 0.07). However, it must be mentioned that leukemic lymphocytes in the peripheral blood of studied patients with CLL belong to B-cell lineage in contrast to the blood donors who possess mainly T- lymphocytes. On the other hand, T as well as B mature lymphocytes contain the same nucleolar types and mainly ring shaped nucleoli [24].

The nucleolar and cytoplasmic RNA image density (Table 2) The nucleolar and cytoplasmic RNA image density appeared to be very stable in both immature and mature lymphocytes of untreated as well as treated patients since the measured values and resulting nucleolar to cytoplasmic RNA image density ratios were almost the same.

Gumprecht ghosts and apoptotic cells and bodies of lymphocytic origin (Table 2)

Gumprecht ghosts always possessed distinct nucleoli bodies (Fig. 4a). Most of Gumprecht ghosts contained ring shaped nucleoli or micronucleoli and thus, originated from mature lymphocytes (Fig 4a). Nucleoli were also present in both apoptotic lymphocytes (Fig 4b) and apoptotic bodies (4c) with characteristic nuclear changes [4, 25, 26]. On the other hand, the presence of apoptotic cells or bodies in the peripheral blood was exceptional.

Table 2. The nucleolar (No) and cytoplasmic (Cy) RNA image density (RNAIDn), the nucleolar diameter (NoDm in μm)

Cells	Therapy	NoRNAIDn	CyRNAIDn	No/Cy RNAIDn	NoDm
Lybl	0	**81.0** ± 6.9	**72.4** ± 8,6	**1.1**	**2.14** ± 0.11
	+	**92.5** ± 10.8	**77.6** ± 11.9	**1.2**	**2.09** ± 0.33
Lycyt	0	**82.1** ± 9.5	**77.9** ± 14.3	**1.0**	**1.21** ± 0.10§
	+	**82.4** ± 11.1	**68.2** ± 10.0$^{\&}$	**1.2**	**1.04** ± 0.07§†
Gumprecht ghosts	–	**78.7** ± 11.9	**44.5** ± 24.0*	**1.8**	**2.14** ± 0.32$^{\square}$
Apo ly + apo bodies	–	**91.2** ± 7.1	**66.0** ± 5.3*$^{\#}$	**1.4**	**1.04** ± 0.10$^{\Delta}$

Legend
* – significantly different from lymphocytes using t-test (p<0.03)
$^{\#}$ – significantly different from Gumprecht ghosts using t-test (p<<0.01)
§ – significantly different from lymphoblasts using t-test (p<0.001)
$^{\square}$ – significantly different from lymphocytes using t-test (p<0.001)
$^{\Delta}$ - significantly different from Gumprecht ghosts using t-test (p<0.001)
† – significantly different from lymphocytes of untreated patients with cytostatics using t-test (p<0.001)
$^{\&}$ – significantly different from lymphoblasts of treated patients with cytostatics using t-test (p<0.01)
Apo ly + apo bodies – apoptotic lymphocytes + apoptotic bodies
For other legend see Fig. 1

Nucleoli in Gumprecht ghosts were apparently swollen and significantly enlarged in comparison with intact mature lymphocytes (Fig 4a). In contrast to Gumprecht ghosts, nucleoli in a limited number of apoptotic cells not exhibit a nucleolar enlargement (Fig. 4b).

It should be also noted that the nucleolar to cytoplasmic RNA image density ratio in Gumprecht ghosts was markedly elevated because the smeared cytoplasm lost most of the RNA. However, the nucleolar RNA image density in Gumprecht ghosts was only slightly decreased. The nucleolar swelling was very variable and the increased values of the nucleolar RNA image density were not significant (the variation coefficient reached 15%) in comparison with intact lymphocytes. Similar, but much smaller increase of the nucleolar to cytoplasmic RNA image ratio was also observed in apoptotic lymphocytes in which the cytoplasm did not show a substantial loss of RNA.

Discussion

The present study provided complementary information on nucleoli in differentiating and maturing leukemic B-lymphocytes. The nucleolar and cytoplasmic RNA image density reflecting the nucleolar RNA concentration was very stable and did not show substantial differences between early and mature stages of the lymphocytic development. In contrast to granulopoiesis, such stability was also noted during the erythroid differentiation [12, 13]. Since the nucleolar diameter markedly decreased in mature and terminal lymphocytes, it seems to be likely that the decreasing RNA content in nucleoli

during lymphocytic maturation was apparently related to the nucleolar size. The significantly decreased cytoplasmic and slightly reduced nucleolar RNA concentrations together with reduced nucleolar size in mature leukemic B- lymphocytes are also in harmony with such conclusion. The decreased RNA values in leukemic mature B-lymphocytes were also reported previously using flow cytometry, but without respect to the therapy of studied patients [27]. At this connection it was presumed that most of reduced RNA content was represented by rRNA transcribed in the nucleolus.

As it was expected, immature lymphocytes mostly possessed large nucleoli, which were in mature lymphocytes replaced by ring shaped nucleoli and micronucleoli. The cytostatic therapy resulted in the increased incidence of micronucleoli in both immature and mature lymphocytes [28]. Such change was also accompanied by an increase of the nucleolar coefficient, i.e. number of nucleoli per cell. Such effect of the cytostatic therapy was not surprising and just indicated that active large nucleoli with multiple small fibrillar centers and resting nucleoli with one large fibrillar centers were replaced by inactive micronucleoli. Unfortunately, it must be mentioned that „large active" and „ring shaped resting" nucleoli were still present in immature lymphocytes after the cytostatic therapy. Thus such cells might represent a potential pool for proliferation. It shoud be added that leukemic lymphocytes with large nucleoli were also considered to be unfabourable prognostic markers for patients resistant to the chemotherapy. [29]. „Large active nucleoli" with multiple fibrillar centers are known to be characteristic for proliferating cells [5, 7]. „Ring shaped resting nucleoli" are characteristic for sleeping cells, which may be stimulated to return to the cell cycle and proliferate again [see 1, 5, 17].

A markedly enlarged nucleolar to cytoplasmic RNA image density ratio was noted in Gumprecht ghosts. Such increase was apparently due to the marked loss of the cytoplasmic RNA during smear preparations [see 12]. The presence of swollen ring shaped nucleoli and distinct micronucleoli apparently indicated that Gumprecht ghosts mainly originate from mature lymphocytes. The increased nucleolar to cytoplasmic RNA image ratio was also noted in apoptotic lymphocytes or bodies. However, the decrease of the cytoplasmic RNA image density was much smaller than in Gumprecht ghosts. In apoptotic lymphocytes and apoptotic bodies of the lymphocytic origin the nucleolar RNA image density "was frozen" since it was practically the same as in morphologically intact immature or mature lymphocytes. The „frozen RNA" in nucleoli of apoptotic cells and bodies has been already described previously under experimental conditions that induced the apoptotic process in leukemic myeloblasts [30]. Thus the high nucleolar RNA image density values observed in the present study are in harmony with such observations.

It seems to be also interesting that nucleolar to cytoplasmic RNA image density ratio in Gumprecht ghosts and apoptotic lymphocytes to some extent resembled that in last stages of the granulocytic development. In advanced stages of the granulocytic lineage the similarly increased nucleolar to cytoplasmic RNA image density ratio was also due to the gradual loss of the cytoplasmic RNA [13].

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