NUCLEOLAR MORPHOLOGY AND MATURATION OF THYMIC LYMPHOCYTES

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INTRODUCTION

The thymus is considered to be the primary center for the differentiation of immunologically committed cells. There is a rapid turnover of cells in the thymus involving continuous entry of new stem cells from extrathymic sources. These cells proliferate, mature, and differentiate, and finally are either destroyed or migrate into the circulation (2, 7). Therefore, the thymus can serve as a convenient model for studies of cell proliferation and differentiation. Nucleoli in immature lymphocytes are usually compact (dense) with nearly homogeneous distribution of ribonucleoproteins or with defined trabecular structures separated by light areas (11, 16, 17). Nucleoli in mature lymphocytes are mostly ring shaped and are characterized by the presence of ribonucleoprotein structures in their periphery (15, 17). The ultrastructure of nucleoli was correlated with their light microscope appearance (16, 17). Dense and trabeculate nucleoli are present in cells of the lymphocytic series, with accelerated RNA synthesis after stimulation with phytohemagglutinin; ring-shaped nucleoli seem to reflect a reversible decrease of nucleolar RNA synthesis (13, 19).

In the present study, cells of the lymphocytic series with different types of nucleoli were quantitatively estimated in mouse thymuses. Their nucleolar morphology was correlated with the extent of uridine-5-^{3}H (Ur R-^{3}H) incorporation and with the degree of cell maturation.

MATERIALS AND METHODS

Animals

The experiments were carried out with male mice, 2–3 months old (17.5 ± 2.8 g body weight), of the X/Gf strain (4, 5, 20). Some of the mice were injected intraperitoneally with a single dose of Ur R-^{3}H (Schwarz Bio Research Inc., Orangeburg, N. Y., 2 μCi/g body weight, SA 8 Ci/mmole) and sacrificed 45 min later. This time interval was found optimal, in preliminary experiments, for an efficient labeling of thymic cells in radioautographs. Mice were sacrificed by disconnecting the spinal cord, and thymuses were immediately removed.

Light Microscope Preparations

Cross-sections of thymic lobes fixed in 4% neutral formaldehyde solution were routinely processed and cut at 5 μ. Other cross-sections of lobes were used for contact preparations. The preparations were stained either with May-Grünwald Giemsa's or with 0.05% solution of toluidine blue at pH 5.0 (15, 18). Other contact preparations, fixed in methyl alcohol, were used for radioautography. Histologic sections and some contact preparations were covered with Kodak Nuclear Track Emulsion NTB_2 and stored at 4°C for 105 days. After developing, the sections were stained through the emulsion with 0.1% solution of toluidine blue pH 5.0, and contact preparations, with 0.05% solution (12).

Criteria and Statistical Evaluation

The proportion of lymphoblasts, prolymphocytes, and mature lymphocytes in thymuses was estimated by differential count in contact preparations stained with May-Grünwald Giemsa's. Percentages of these cell types were calculated for subsets of 100 cells, and cumulative curves of percentages were examined as the functions of the number of cells (14). The range of variations decreased to less than 5% or 1% after 700–1600 counts. 2000 thymic lymphocytes were counted in each thymus.

The distribution of RNA-containing structures in nucleoli was evaluated in cells of the lymphocytic series, using contact preparations stained with toluidine blue. Nucleoli were classified in 50 or in 100 lymphoblasts and prolymphocytes and in 100 or 200 mature lymphocytes of each thymus. The results were expressed as the percentage of cells with various types of nucleoli and as the percentage of nucleolar types per se present in cells with differing degree of maturity. The size of nucleoli was measured, in some preparations, on a calibrated ocular grid.

The differential count of cells in radioautographs of histologic sections was evaluated in terms of large (> 10 μ in diameter), medium (6–10 μ), and small (< 6 μ) lymphocytes. In each sample, silver grains were counted over 100 cells of each cell type, separately, in thymic cortex and medulla. The labeling of cells with different types of nucleoli was evaluated in radioautographs of contact monolayer preparations. 100 lymphoblasts and prolymphocytes and 200 mature lymphocytes were evaluated in each sample. The effect of background was considered in statistical evaluation with the use of grain count distribution curves (9), Stillström's method (1), and grain count over empty spaces without cell structures and debris. The results obtained were evaluated and compared, using the standard deviation of the mean values, the standard deviation of the measurements in pairs, the correlation coefficient, and Student's test (3, 8).

RESULTS

The proportion of lymphoblasts, prolymphocytes, and mature lymphocytes in thymuses of X/Gf mice is shown in Table I. Three different types of nucleoli were present in cells of the lymphocytic series stained with toluidine blue: dense, trabeculate, and ring shaped (Fig. 1). Dense nucleoli were observed only in lymphoblasts (5.4 ± 2.7% of all nucleoli present in lymphoblasts), large trabeculate nucleoli (> 4 μ in diameter) prevailed in lymphoblasts (54.5 ± 10.4%), and the highest incidence of trabeculate nucleoli < 4 μ in diameter was noted in prolymphocytes (36.2 ± 4.0%). Ring-shaped nucleoli predominated in prolymphocytes and mature lymphocytes (56.2 ± 4.6 and 78.1 ± 5.1% of all nucleoli). The mean number of nucleoli per cell was higher in lymphoblasts (4.61) and prolymphocytes (3.72) than in mature lymphocytes (1.52).

Thymic cells of the lymphocytic series were divided according to their nucleolar morphology: (*a*) *Cells with dense nucleoli;* some of these cells also possessed trabeculate and ring-shaped nucleoli; (*b*) *cells with trabeculate nucleoli;* a certain portion of these cells also possessed ring-shaped nucleoli; (*c*) *cells with ring-shaped nucleoli.* The quantitative evaluation of all three cell types is presented in Table I.

The grain counts over cells of the lymphocytic series were compared between cortex and medulla

TABLE I

Cells of the Lymphocytic Series in Mouse Thymuses

Cell types*	% ± SD	Range of variation‡	Cells with dense nucleoli	Cells with trabeculate nucleoli	Cells with ring-shaped nucleoli
			% ± SD§	% ± SD	% ± SD
Lymphoblasts	3.81 ± 0.71	<1%, <5%	11.2 ± 2.2	84.7 ± 5.1	4.1 ± 2.1
Prolymphocytes	8.70 ± 0.91	<1%, <5%	—	70.5 ± 5.5	29.5 ± 5.5
Mature lymphocytes	87.47 ± 1.58	<1%	—	34.7 ± 5.3	65.3 ± 4.9

* Differential count of cells in thymuses of 10 X/Gf mice. Contact preparations were stained with May-Grünwald Giemsa's; 2000 cells were evaluated in each thymus.

‡ Percentages of all cell types were calculated for subsets of 100 cells, and cumulative curves of percentages were examined as the function of the number of cells (Simard and Daoust, 1966). The indicated range of variations was reached after 700–1600 counts.

§ Cells with different types of nucleoli in thymuses of 10 X/Gf mice. Contact preparations were stained with toluidine blue; 50 or 100 lymphoblasts and prolymphocytes, and 100 or 200 mature lymphocytes were evaluated in each thymus; 3.4 ± 0.9% lymphoblasts with dense nucleoli also possessed ring-shaped nucleoli; 9.3 ± 2.1% lymphoblasts, 16.9 ± 6.3% prolymphocytes, and 9.2 ± 4.7% mature lymphocytes with trabeculate nucleoli also possessed ring-shaped nucleoli.

in three thymuses labeled with Ur R-^{3}H, using radioautographs of histologic sections. The coefficient of correlation between the grain counts over large, medium, and small lymphocytes is highly significant (r = 0.9708–0.9976, 1 d.f., $P < 0.005$ or $P < 0.001$ for a two tail test). The number of labeled cells of these types in cortex and medulla was compared, using the standard deviation of the measurement in pairs. This correlation indicated that the deviation for all three types of cells was close to the confidence level of two sigma.

The distribution of silver grains was compared among cells of the lymphocytic series with different types of nucleoli, using radioautographs of contact preparations from three thymuses. After a 45 min pulse, grains were located mostly over dense and large trabeculate nucleoli and over adjacent nucleoplasm; cells with trabeculate nucleoli showed a lower labeling, and cells with ring-shaped nucleoli a very low or no labeling at all (Fig. 1). The grain count over cells and over nucleoli was recorded as grain count distribution curves for lymphoblasts, prolymphocytes, and mature lymphocytes (Fig. 2). The mean number of grains over lymphoblasts with ring-shaped nucleoli was approximately 6 times lower, over prolymphocytes with ring-shaped nucleoli 4 times lower, and over mature lymphocytes with ring-shaped nucleoli 3 times lower than the mean number of grains over corresponding cells with dense or trabeculate nucleoli. These differences are statistically significant ($P < 0.01$, $P < 0.01$, and $P < 0.02$, respectively). The differences between the number of grains over cells with dense or trabeculate nucleoli and the number of grains over cells with both trabeculate and ring-shaped nucleoli are not significant ($P > 0.05$).

DISCUSSION

The primary objective of this study was to establish a quantitative relationship between nucleolar morphology and maturation of thymic lymphocytes. Nucleoli with homogeneous distribution of ribonucleoprotein structures (dense nucleoli) and with trabecular structures (trabeculate nucleoli) predominated in lymphoblasts, whereas ring-shaped nucleoli prevailed in mature lymphocytes. The approximate size of nucleoli, expressed in terms of large trabeculate nucleoli (> 4 μ in diameter) and trabeculate nucleoli < 4 μ in diameter, decreased in prolymphocytes and mature lymphocytes. The quantitative evaluation of cells with different types of nucleoli and of nucleoli per se suggests that, during the progressive maturation of lymphocytes, nucleoli, in a majority of cells, changed from "immature" dense and large trabeculate to trabeculate and finally to "mature" ring shaped. This conclusion correlates with observations made on leukemic lymphocytes (16, 17). A reverse process, i.e. a conversion of ring-shaped nucleoli to trabeculate and dense, was observed in lymphocytes after stimulation with phytohemag-

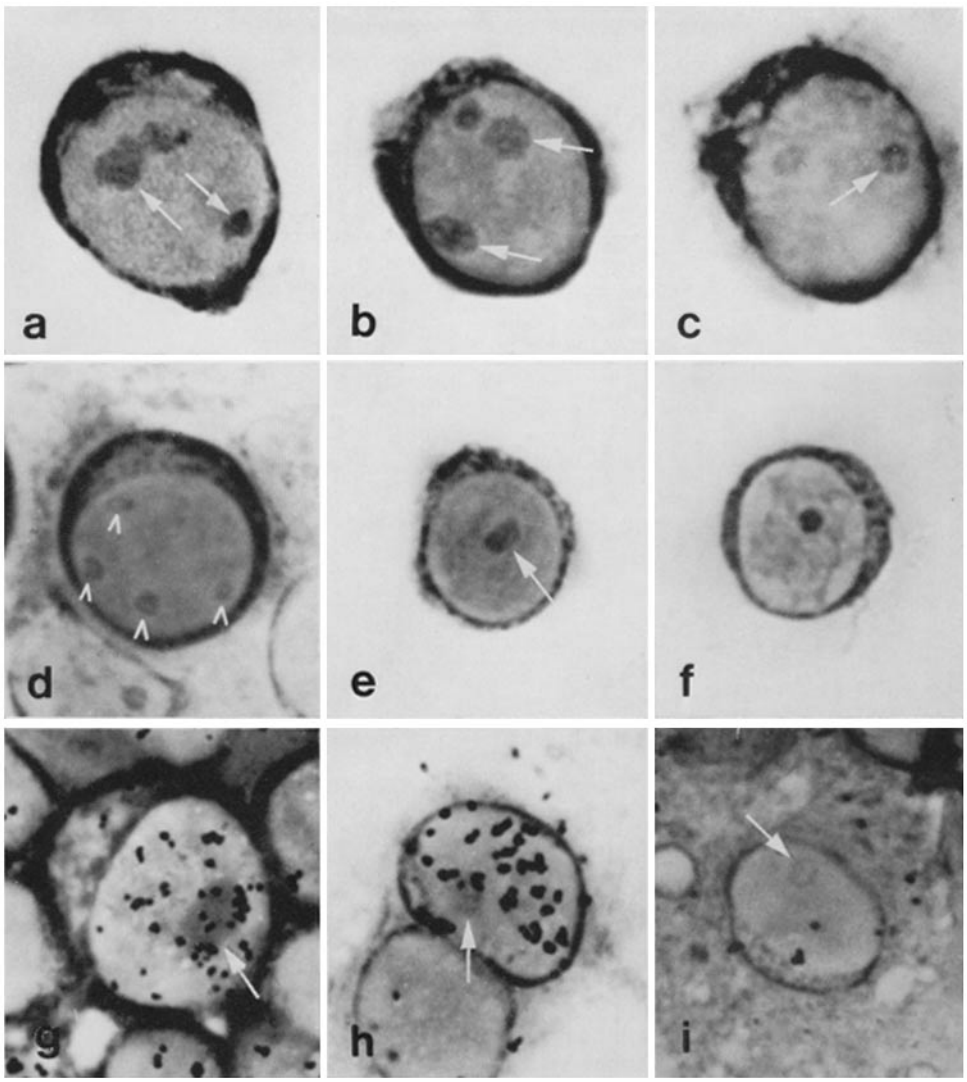

FIGURE 1 *Lymphoblasts* (*a*) with dense nucleoli (arrows); (*b*) with trabeculate nucleoli (arrows); (*c*) with ring-shaped nucleolus (arrow). *Prolymphocyte* (*d*) with ring-shaped nucleoli (points). *Mature lymphocytes* (*e*) with trabeculate nucleolus (arrow), (*f*) with ring-shaped nucleolus. *Radioautographs* (uridine-5-^{3}H labeling) (*g*) lymphoblast—silver grains over dense nucleolus (arrow) and nucleus, (*h*) prolymphocyte—silver grains over trabeculate nucleolus (arrow) and nucleus; (*i*) mature lymphocyte with ring-shaped nucleolus (arrow)—silver grains are rare. Contact preparations and radioautographs stained with toluidine blue. × 2500.

glutinin (10, 13). A small portion of lymphoblasts (4.1%) and less than one-third of prolymphocytes contain only ring-shaped nucleoli. The structure of ring-shaped nucleoli in these cells is similar to the structure of nucleoli observed in mature lymphocytes. The number of nucleoli per cell decreased gradually during maturation of thymic lymphocytes, as was noticed in other cell types (6, 21).

Nucleolar morphology of thymic lymphocytes was correlated with quantitative radioautography using Ur R-^{3}H as the tracer. Lymphoblasts and prolymphocytes with dense and large trabeculate nucleoli, rich in ribonucleoproteins, exhibited

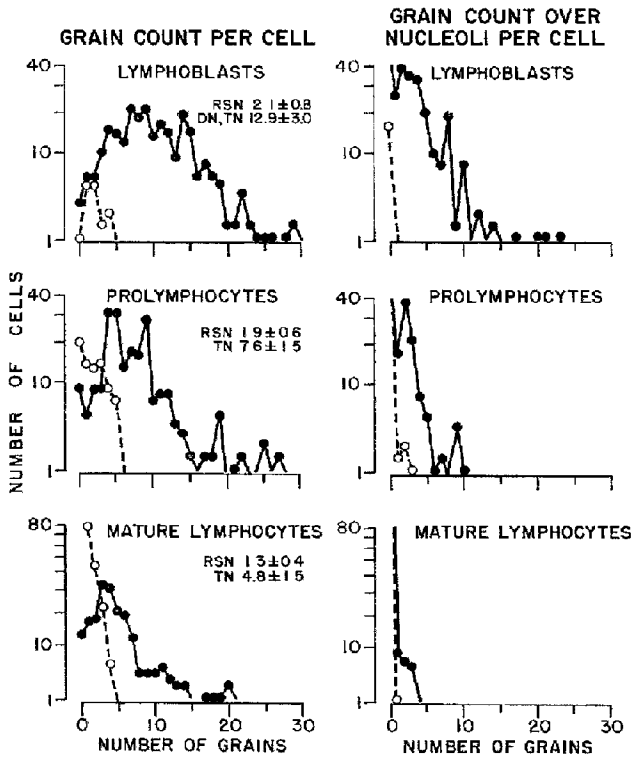

FIGURE 2 Incorporation of Ur R-^{3}H in cells of the lymphocytic series (●——●, cells with dense and trabeculate nucleoli; ○---○, cells with ring-shaped nucleoli); 100 lymphoblasts, 100 prolymphocytes, and 200 mature lymphocytes were evaluated for the grain count and nucleolar morphology in each set of contact preparations of three thymuses. The background corresponded to 0.39–0.42 grains/100μ^2 Mean number of grains over cells with dense nucleoli (*DN*), over cells with trabeculate nucleoli (*TN*) and over cels with ring-shaped nucleoli (*RSN*) are included in the chart.

intensive incorporation. Mature lymphocytes with ring-shaped nucleoli showed a very low incorporation of Ur R-^{3}H, which seems to indicate a decrease in nucleolar RNA synthesis, but mature lymphocytes with trabeculate nucleoli incorporated Ur R-^{3}H These cells probably preserved a higher rate of RNA synthesis as compared with mature lymphocytes possessing ring-shaped nucleoli. The difference in incorporations of the tracer between both groups is significant ($P <$ 0 02). The presence of ring-shaped nucleoli in lymphoblasts and prolymphocytes may indicate that these cells are in "resting" stage Their incorporation of the tracer was limited and was significantly lower than in their counterparts with dense or trabeculate nucleoli ($P <$ 0 01). The relevance of immature cells of the lymphocytic series with ring-shaped nucleoli is, in respect to cell proliferation kinetics, rather conjectural and remains to be elucidated

SUMMARY

Maturation and differentiation of lymphocytes in mouse thymuses was accompanied by morphologic changes of nucleoli. This was observed on contact preparations stained with toluidine blue. Cells possessing dense nucleoli with homogeneous distribution of basophilic ribonucleoprotein structures or nucleoli with trabecular structures separated by light areas constituted 95.9% of all lymphoblasts. Cells with trabeculate nucleoli constituted 70.5% of prolymphocytes and 34.7% of mature lymphocytes Large trabeculate nucleoli ($> 4\ \mu$ in diameter) predominated in lymphoblasts. A small portion of thymic lymphoblasts (4 1%) contained only ring-shaped nucleoli with ribonucleoproteins located in their periphery. Cells with ring-shaped nucleoli represented 29.5% of prolymphocytes and 65.3% of mature lymphocytes

In radioautographs, lymphoblasts with dense and large trabeculate nucleoli rich in ribonucleoproteins exhibited intensive labeling with uridine-5-^{3}H. Prolymphocytes and mature lymphocytes with trabeculate nucleoli showed lower labeling, and immature or mature lymphocytes with ring-shaped nucleoli showed very low or no labeling. Differences between the mean number of grains over immature or mature lymphocytes with dense or trabeculate nucleoli and the mean number of grains over immature or mature lymphocytes with ring-shaped nucleoli are significant ($P < 0.01$, $P < 0.02$). These findings suggest (*a*) gradual restriction of the nucleolar function with respect to RNA synthesis in the maturative process of the majority of thymic lymphocytes, and (*b*) the existence of immature lymphocytes with reduced nucleolar function.

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