Nucleolar organizer regions: their significance in the maturation of the gut-associated lymphoid tissue

D. Tamiolakis[1], A. Kotini[2], M. Lambropoulou[3], I. Tolparidou[3], N. Papadopoulos[4]

[1]Department of Cytology, Regional Hospital of Alexandroupolis
[2]Department of Medical Physics, Democritus University of Thrace
[3]Department of Pathology Democritus University of Thrace
[4]Department of Histology-Embryology, Democritus University of Thrace, Alexandroupolis (Greece)

Summary

Nucleolar organizer regions (NORs) are important for regulating protein synthesis. Our study points towards the possibility of determining gut-associated lymphoid (GAL) cell proliferation and maturation in fetuses from ten to 35 weeks of gestation by means of the Argyrophilic (Ag) staining for NORs. This technique was performed on paraffin-embedded small intestine and mesentery tissue sections from 30 fetuses at the 10th, 14th, 15th, 19th, 20th, 23rd, 31st, and 35th week of gestation.

Our results showed that there was no statistically significant difference concerning the mean AgNOR values, between GAL cells and mesentery lymphoid cells (2.81 ± 0.63 to 2.63 ± 0.71; $p > 0.1$), from ten to 15 weeks' gestation. In this specific period, the lymphoid cells first appear and Peyer's patches are formed in the small intestinal mucosa. In contrast, in fetuses from 19 to 35 fetal weeks NOR counts were statistically significantly greater in GAL cells than in fetuses from ten to 15 gestational weeks (as compared to NOR counts in GAL cells in fetuses at an earlier fetal age) (3.28 ± 0.73 to 2.81 ± 0.63). Mean AgNOR values in mesentery lymphoid cells remained unchanged (2.62 ± 0.57) ($p < 0.001$).

In conclusion, increasing protein synthesis from the 19th till the 35th week of gestation is indicative of immune system maturation, given that at the same time GAL cells become fully mature.

Mesentery lymphoid cells pass (migrate) to the small intestinal mucosa from the bloodstream, and they form the whole out-associated lymphoid tissue (GALT) organ (Peyer's patches). The lymphatics associated with mucosa associated lymphoid tissue (MALT) are all efferent; it seems that during fetal life due to genetically determined factors, draining vessels operating as afferent lymphatics at that time, change to efferent (where the immunological response is greatly amplified) as embryonal life progresses.

Key words: Nucleolar organizer; Lymhoid tissue.

Introduction

The lymph vascular system originates from mesenchyme; as does the whole circulatory system [1]. At the tenth fetal week lymphoid elements appear in the alimentary tract, at the 14th week lymphoid aggregates in the form of follicles with germinal centers appear, and finally at the 15th fetal week Peyer's patches are found in the small intestinal mucosa [2, 3]. The total mass of lymphoid tissue is now considered to be a lymphoid organ in its right, and is collectively known as GALT. The larger aggregations function in a manner analogous to lymph nodes, sampling antigenic material entering the tracts and mounting both antibody-mediated and cytotoxic immune responses where appropriate.

The aim of our study was to investigate the possibility of determining the maturation level of the intestinal immune system in fetuses from ten to 35 weeks' gestation; also to find out the origin of lymphoid aggregations distributed throughout the mucosa and to be more specific in determining whether they migrate from mesentery or other distant sites or if they develop from mesenchymal mesodermal cells of the visceral layer covering the primitive gut. Conseguently, we investigated more objective methods. A newfound method to evaluate cellular activity in a variety of neoplastic or hyperplastic disorders is that of NORs which regulate protein-synthesis.

Revised manuscript accepted for publication November 4, 2001

NORs are loops of DNA which are responsible for ribosomal RNA (rRNA) transcription [4]; they can be readily identified in paraffin wax sections by means of a silver (Ag) staining technique (AgNOR). They are visualized in the nuclei of cells as black dots. The AgNOR technique has been used by cytogenetistics for the appraisal of genetic abnormalities, especially trisomy 21 [5]. It was modified by Ploton *et all.* [6], and today is applied in histopathology research in assessing neoplastic or hyperplastic processes and the results in general show different (AgNOR) counts in malignant and benign cells.

The drawback of this method is the definition of NOR [7]. Some authors consider NOR as, the entire nucleolus, although there are discrete dots in it [8, 10]. Others consider NOR as each individual dot inside the nucleolus as well as each dot outside the nucleolus and inside the nucleus [7, 11, 12, 13]. Both methods of measuring NORs have advantages and disadvantages. Those who support the first method believe that measuring NORs inside the nucleolus is subjective and not reproducible [8, 10]. Those who support the second method believe that only the total number of NORs can be used in distinguishing proliferating from non-proliferating cells [14, 15]. In our work we consider NORs as only extra-nucleolar dots.

Even though there are a great number of comparative studies of AgNORs in the literature in various benign and malignant lesions of the lymphoid tissue [9, 16, 17, 18,

19], we did not find references about the maturation state and the origin of intestinal mucosa-associated lymphoid tissue that is based on this method.

Materials and Methods

Our material was obtained from 30 fetuses at various stages of development (10th, 14th, 15th, 19th, 20th, 23rd, 31st, and 35th weeks) selected from the routine histological files, and consists of histological sections from the small intestine and the proper mesentery.

Our subject matter was divided into 12 groups and a comparable analysis of the results was performed.

1st group: Lymphoid cells from the small intestinal mucosa (10th, 14th and 15th weeks)

2nd group: Lymphoid cells from the mesentery (10th, 14th and 15th weeks)

3rd group: Lymphoid cells from the small intestinal mucosa (19th, 20th and 23rd weeks)

4th group: Lymphoid cells from the mesentery (19th, 20th and 23rd weeks)

5th group: Lymphoid cells from the small intestinal mucosa (31st and 35th weeks)

6th group: Lymphoid cells from the mesentery (31s and 35th weeks)

7th group: Mesenchymal cells surrounding lymphoid aggregations in the lamina propria of the small intestinal mucosa (10th, 14th and 15th weeks)

8th group: Mesenchymal cells from the mesentery surrounding lymphoid aggregations (10th, 14th and 15th weeks)

9th group: Mesenchymal cells surrounding lymphoid aggregations in the lamina propria of the small intestinal mucosa (19th, 20th and 23rd weeks)

10th group: Mesenchymal cells from the mesentery surrounding lymphoid aggregations (19th, 20th and 23rd weeks)

11th group: Mesenchymal cells surrounding lymphoid aggregations in the lamina propria of the small intestinal mucosa (31st and 35th weeks)

12th group: Mesenchymal cells from the mesentery surrounding lymphoid aggregations (31st and 35th weeks)

The AgNOR technique, as described by Smith and Crocker was applied to paraffin wax sections of 3 μm. In brief, the sections were dewaxed in xylene, hydrated through graded ethanol, and washed with deionised water. They were exposed to freshly prepared AgNOR staining solution: one volume of a 2% solution of gelatin in 1% formic acid mixed with two volumes of 50% aqueous silver nitrate solution. The reaction was performed at room temperature in the dark for 30 min. The silver colloid was then washed off with running deionised water and sections were dehydrated through alcohol to xylene and mounted in synthetic medium.

AgNORs were measured in 100 cells as suggested by Howat's group [8]. In this procedure only discrete, easily discernible, black dots are enumerated. Cells were examined using a x100 oil immersion objective. Microscopical fields were randomly selected. The analysis of the results was performed by the Student's unpaired t-test.

Three [3] observers performed AgNOR counts and no significant divergences were noted.

Results

Our results are shown in Diagrams 1 and 2. AgNORs were observed in almost the majority of the nuclei of the lymphoid and the mesenchymal cells in all embryonal tissues examined at different weeks of development (Figure 1).

Diagram 1

1st group: AgNOR mean value in the lymphoid cells of the intestinal mucosa was 2.81 ± 0.63 (1.8-3.7) (at the 10th, 14th, and 15th week of gestation).

2nd group: AgNOR mean value in the lymphoid cells of the mesentery was 2.63 ± 0.71 (1.8-3.9) (at the 10th, 14th, and 15th week of gestation).

3rd group: AgNOR mean value in the lymphoid cells of the intestinal mucosa was 3.28 ± 0.73 (2.4-4.5) (at the 19th, 20th, and 23rd week of gestation).

4th group: AgNOR mean value in the lymphoid cells of the mesentery was 2.62 ± 0.57 (1.8-3.5) (at the 19th, 20th, and 23rd week of gestational).

5th group: AgNOR mean value in the lymphoid cells of the intestinal mucosa was 3.49 ± 0.67 (2.9-4,6) (at the 31st and 35th week).

6th group: AgNOR mean value in the lymphoid cells of the mesentery was 2.64 ± 0.61 (1.7-3.4) (at the 31st and 35th week).

Diagram 2

7th group: AgNOR mean score in the mesenchymal cells of the intestinal mucosa was 2.43 ± 0.62 (1.5-3.7) (at the 10th, 14th, and 15th week of gestation).

8th group: AgNOR mean score in the mesenchymal cells of the mesentery was 2.65 ± 0.73 (1.7-3.7) (at the 10th, 14th, and 15th week of gestation).

9th group: AgNOR mean score in the mesenchymal cells of the intestinal mucosa was 2.47 ± 0.49 (1.8-3.4) (at the 19th, 20th, and 23rd week of gestation).

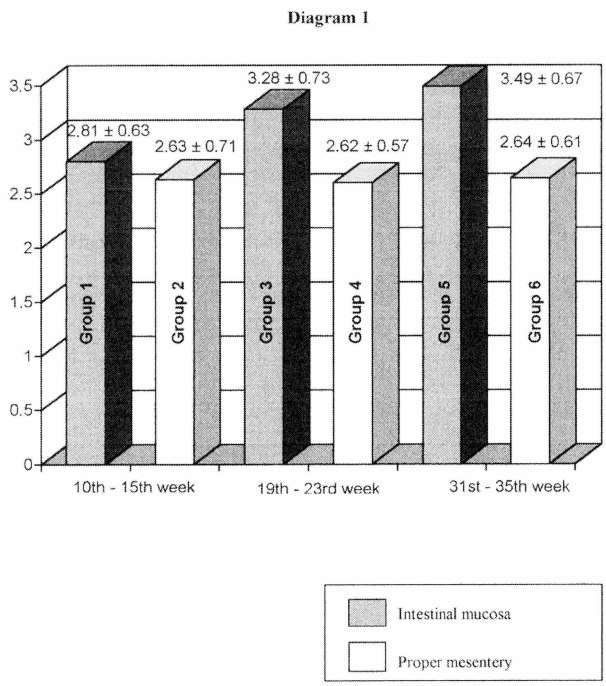
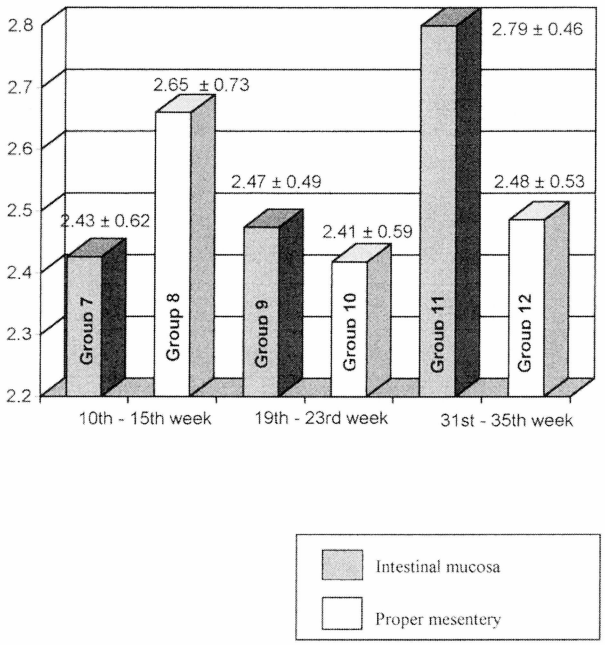


Diagram 2



10th group: AgNOR mean score in the mesenchymal cells of the mesentery was 2.41 ± 0.59 (1.6-3.7) (at the 19th, 20th, and 23rd week of gestation).

11th group: AgNOR mean score in the mesenchymal cells of the intestinal mucosa was 2.79 ± 0.46 (1.7-3.5) (at the 31st and 35th week).

12th group: AgNOR mean score in the mesenchymal cells of the mesentery was 2.48 ± 0.53 (1.7-3.3) (at the 31st and 35th week).

A comparative study between the groups was performed and the statistical analysis showed:

1. No statistically significant difference in the AgNOR mean value between lymphoid cells of the intestinal mucosa (group 1) and the lymphoid cells of the mesentery (group 2) till the 15th fetal week ($p > 0.1$).

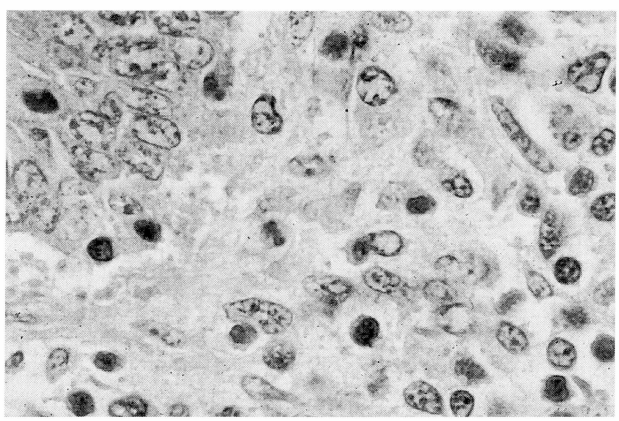


Figure 1. — AgNORs are observed in almost the majority of the nuclei of the lymphoid and the mesenchymal cells (AgNOR stain x250).

2. A statistically very significant difference in the AgNOR mean value between lymphoid cells of the intestinal mucosa (groups 3, 5) and lymphoid cells of the mesentery (groups 4, 6) from the 19th till the 35th fetal week ($p < 0.001$).

3. No statistically significant difference in the AgNOR mean value between mesenchymal cells of the intestinal mucosa (group 7) and the mesenchymal cells of the mesentery (group 8) from the 10th till the 15th fetal week ($p > 0.1$).

4. No statistically significant difference in the AgNOR mean value between intestinal intraepithelial mesenchymal cells (group 9) and mesenchymal cells of the mesentery (group 10) from the 19th till the 23rd fetal week ($p > 0.1$).

5. A statistically significant difference in the AgNOR mean value between intestinal intraepithelial mesenchymal cells (group 11) and mesenchymal cells of the mesentery (group 12) from the 31st till the 35th fetal week ($p > 0.01$).

Discussion

One of the various methods of evaluating the cell potential is the recognition and measurement of NORs after the ascertaining that their number is greater in activated than in normal cells.

The NOR scoring was applied in a great number of cases of benign, malignant and borderline lesions in the lymphoid tissue, breast, large intestine, nose, cervix, endometrium, ovaries, salivary glands, as well as in melanocytic tumors of the skin.

In the field of continuing reseach on NORs, and considering the clinical practice of NOR scoring, embryology is strongly involved, given that a lot of conclusions may arise from the NOR values in proliferating cells concerning the maturation stage and their functional skills.

In this study and towards an attempt to determine the maturation process and origin of the gut-associated lymphoid tissue, we applied NOR scoring in lymphoid and mesenchymal cells of the proper mesentery and the small intestinal mucosa from fetuses examined at different stages of development. In this way we wanted to record: 1) The different counts of NORs in lymphoid cells of the intestinal mucosa at different fetal ages. 2) The different counts of NORs in lymphoid cells of the intestinal mucosa and lymphoid cells of the islands of lymphoid tissue associated with the proper mesentery, considering that a statistically significant difference in NOR counts in these separate cell populations at early fetal ages would be indicative of a different origin. 3) The different counts of NORs in mesenchymal cells surrounding the lymphoid cells of the intestinal mucosa and mesenchymal cells surrounding the islands of lymphoid cells of the proper mesentery, in order to evaluate and compare if the intraepithelial intestinal lymphoid cells in these sites originate from intestinal mesenchymal cells, due to genetically determined factors.

Our findings showed: 1) No statistically significant difference in the NOR mean value between lymphoid cells

of the intestinal mucosa (2.81 ± 0.63) and islands of lymphoid tissue of the mesentery (2.63 ± 0.71) ($p > 0.1$) from 10 to 15 weeks' gestation, timing of the first appearance of lymphoid cells and the first Peyer's patch formations in the small intestinal mucosa. On the contrary, in fetuses from 19 to 35 weeks, NOR numbers were significantly greater in intestinal intraepithelial lymphoid cells (3.28 ± 0.73), as compared to NOR numbers in lymphoid cells of the intestinal mucosa in fetuses of fewer weeks (10-15) (2.81 ± 0.63). As far as the mean NOR value, in the lymphoid cells of the mesentery is concerned, it did not change (2.62 ± 0.57) ($p < 0.001$).

We conclude that, increased protein synthesis from 19 to 35 weeks' gestation is indicative of intestinal immune system development, considering that at this same time period gut-associated lymphoid tissue has been fully structurally organized.

2) The results also showed no statistically significant difference in the NOR mean value in the lymphoid cells of the intestinal mucosa and the mesentery from the 10th to 15th fetal weeks ($p > 0.1$), the time of intestinal intraepithelial lymphoid tissue appearance. We conclude that lymphoid cells recognized in the intestinal mucosa from 10th to 15th fetal week originate from the same cell population seen in the proper mesentery and then migrating into the intestinal mucosa. On the contrary, a statistically significant difference was noted in the NOR mean value between lymphoid cells of the intestinal mucosa and lymphoid cells of the mesentery from the 19th to 35th week of development ($p < 0.001$), the time of intestinal immune system development (structural organization), also known as gut-associated lymphoid tissue (GALT).

3) Finally, we found no statistically significant difference in the NOR mean value between mesenchymal cells in the intestinal mucosa and the mesentery from the 10th till the 23rd week of gestation ($p > 0.1$). In this period, mucosa-associated lymphoid tissue first appears and subsequently Peyer's patches are formed throughout the gut. On the contrary, we observed a statistically significant difference in the NOR mean value between mesenchymal cells of the intestinal mucosa and mesentery, from the 31st to 35th fetal week ($p < 0.001$), the time of intense development of connective tissue supporting the vigorously organizing gut-associated lymphoid tissue.

Thus we conclude that, mesenchymal cells do not differentiate towards lymphoid cells at the time of development of the mucosa-associated lymphoid tissue. The lymphoid cells first appearing in the mucosa at the 10th fetal week originate from lymphoid tissue of the proper mesentery. Considering that the lymphatics associated with MALT are only efferent, it seems that during fetal organogenesis, due to genetically determined factors, draining vessels operating as afferent lymphatics in the above period, transform into efferent lymphatics as embryonal life progresses.

References

[1] Enders A. C., King B. F.: "Formation and differentiation of the extraembryonic mesoderm in the Rhesus monkey". *Am. J. Anat.*, 1988, *181*, 327.
[2] Orlic C., Lev R.: "An electron microscopic study of intraepithelial lymphocytes in human fetal small intestine". *Lab. Invest.*, 1977, *37*, 554.
[3] Butzner J. D., Befus A. D.: "Interactions among intraepithelial leucocytes and other epithelial cells in intestinal development and funtion". In: Lebenthal E. (ed): "Human gastrointestinal development". Raven Press, New York, 1989, 749.
[4] Fakan S., Hernandez-Verdun D.: "The nucleolus and the nucleolar organizer regions". *Biol. Cell.*, 1986, *56*, 189.
[5] De la Cruz F. F., Gerald P. S.: "Trisomy 21". Baltimore: University Park Press, 1981, 165.
[6] Ploton D., Menager M., Jeannesson P., Himper G., Pigeon F., Adnett J. J.: "Improvement in the staining and in the visualization of the argyrophylic proteins of the nucleolar region at the optical level". *J. Histochem.*, 1989, *18*, 5.
[7] Hansen A. B., Ostergard B.: "Nucleolar organizer regions in hyperplastic and neoplastic prostatic tissue". *Virchows Archiv. (Pathol. Anat.)*, 1990, *417*, 9.
[8] Howat A. J., Giri D. D., Cotton D. W. K., Slater D. N.: "Nucleolar organizer regions in spitz nevi and malignant melanomas". *Cancer*, 1989, *63*, 474.
[9] Crocker J., Nar P.: "Nucleolar organizer regions in lymphomas". *J. Pathol.*, 1987, *151*, 111.
[10] Orita T. *et al.*: "Nucleolar organizer regions in meningiomas". *Neurosurgery*, 1990, *26*, 43.
[11] Howat A. J. *et al.*: "Silverstained nucleoli and nucleolar organizer regions counts are of no prognostic value in thick cutaneous malignant melanoma". *J. Pathol.*, 1988, *156*, 227.
[12] Raymond W. A., Leong A. S.: "Nucleolar organizer regions relate to growth fractions in human breast carcinoma". *Hum. Path.*, 1989, *20*, 741.
[13] Coumbe A. *et al.*: "Nucleolar organizer regions in endometrial hyperplasla and neoplasia". *Path. Res. Pract.*, 1990, *186*, 254.
[14] Crocker J., Boldy D. A. R., Egan M. J.: "How should we count AgNORs. Proposals for a standardized approach". *J. Pathol.*, 1989, *158*, 185.
[15] Smith R., Crocker J.: "Evaluation of nucleolar organizer region-associated proteins in breast malignancy". *Histopathology*, 1988, *12*, 113.
[16] Crocker J., Janmohamed R. M. I., Armstrong S. J., Hulten M., Leyland M. J.: "The relationship between numbers of interphase NORs and NOR-bearing chromosomes in non-Hodgkin's lymphoma". *J. Pathol.*, 1989, *157*, 166A.
[17] Crocker J., Macartney J. C., Smith P. J.: "Correlation between DNA flow cytometric and nucleolar organiser region data in non-Hodgkin's lymphomas". *J. Pathol.*, 1988, *154*, 151.
[18] Grotto H. Z. W., Lonard-Metze I., Metze K.: "Nucleolar organiser regions in normal hematopoiesis: relationship to cellular proliferation and maturation". *Nouv. Rev. Hematol.*, 1991, *33*, 1.
[19] Metze K., Lonard-Metze I.: "AgNOR staining in normal bone marrow cells". *J. Clin. Pathol.*, 1991, *44*, 526.

Address reprint requests to:
N. PAPADOPOULOS, M.D.
Ass. Professor in Histology-Embryology,
Democritus University of Thrace.
Dragana, 68 100 Alexandroupolis (Greece)