Moderate and severe malnutrition alters proliferation of spleen cells in rats

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Abstract

Objectives: Previous studies have shown alterations in bone marrow cell proliferation in malnourished rats, during lactation. The objective of this study was to determine *in vivo* effects of moderate and severe malnutrition on spleen cell proliferation in 21-day-old rat pups.

Materials and methods: Spleen cell proliferation was determined following administration of bromodeoxyuridine (BrdUrd) over a time course of 2, 4, 6 and 8 h. Incorporation of BrdUrd was detected using FITC-conjugated anti-BrdUrd monoclonal antibodies and total DNA content was detected and evaluated using propidium iodide using flow cytometry.

Results: Proportions of cells in S and G₂/M were reduced in the rats with moderate (MN2nd) and severe (MN3rd) malnutrition. BrdUrd incorporation was lower in both groups of malnourished rat. In cells of MN2nd individuals, length of G₁ became shorter, while length of S-phase increased. In contrast, fraction of cells in proliferation was significantly lower in both groups of malnourished rat, with MN3rd group having lowest percentage of cell population growth. In this study, severe malnutrition did not significantly affect duration of phases of the cell cycle, although fractions of proliferating cells were dramatically reduced.

Conclusion: Moderate malnutrition increased time of cells in DNA synthesis and time of total cell cycle and severe malnutrition reduced growth fraction of spleen cells in malnourished rats during lactation.

Introduction

Malnutrition has a major negative impact on immune function of children, rendering them more vulnerable to diseases that increase risk of death (1–3). Numbers of authors have suggested that malnutrition is the primary cause of immunodeficiency worldwide (4–6).

In mammals, the spleen is the largest secondary lymphoid organ. Antigens enter the body and circulate to the spleen where lymphocytes are organized into structures that improve cell interactions and promote pathogen removal (7,8). The key function of lymphoid organs is to provide a suitable microenvironment for sequential interaction of different sub-populations of lymphocytes and non-lymphoid cells, to achieve an adaptive immunological response (9,10).

The immune system is compromised in malnourished children and animal models of experimental malnutrition. Atrophy of lymphoid organs has been described in animal models, and the thymus in particular has been termed to be the barometer for malnutrition, by Prentice (11). This phenomenon is due primarily to reduction in lymphoid compartments (12,13). Alterations in other lymphoid tissues, such as the spleen and lymphatic ganglia, have also been observed (14).

In rats, reduction in absolute and relative weight of the spleen and thymus after malnutrition has been induced in the prenatal phase and during lactation (15). In addition to alterations in various cell subpopulations, thymic atrophy has been observed in adults (16). Ortiz *et al.* (17) demonstrated that spontaneous apoptosis is increased in the thymus of malnourished rats during lactation and the same group demonstrated that double-negative thymocytes (CD4[−], CD8[−]) are the most susceptible populations, although increased apoptosis was also observed in CD4⁺ and CD8⁺ subpopulations (18).

In the spleen, CD3⁺ and CD4⁺ lymphocyte subpopulations were shown to be reduced in malnourished rats (19) and in the same study, moderate and severe malnutrition were shown to reduce proportions of CD71⁺ and

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CD25⁺ cells, in cultures stimulated with phytohemagglutinin (PHA). Prolongation of the cell cycle was observed in bone marrow cells of malnourished rats during lactation (20), and reduction in proportions of proliferative cells *in vivo* and *in vitro* have also been shown (21,22). DNA damage has been widely studied in humans and in animal models of malnutrition, and many studies have shown relationships between malnutrition and genetic damage (23,24).

The objective of this study was to determine *in vivo* effects of moderate and severe malnutrition on proliferation of spleen cells in 21-day-old rat pups.

Materials and methods

Experimental malnutrition during lactation

Wistar rats were obtained from a closed breeding colony from the División de Ciencias Biológicas y de la Salud at the Universidad Autónoma Metropolitana, Iztapalapa (UAM-I) in Mexico City, Mexico. They were maintained under 12-h controlled light–dark cycles at temperatures of 22–25 °C with 45% relative humidity. Nursing mothers used in the study had given birth once prior to the litter under study, and here were fed *ad libitum* with a balanced rodent diet (Teklad Global 2018S; Harlan, Madison, WI, USA). Experiments were performed according to guidelines of the Universidad Autónoma Metropolitana (UAM), the Official Mexican Guidelines (Norma Oficial Mexicana NOM-062-ZOO-1999) and the National Institutes of Health (NIH).

Experimental malnutrition was induced by food competition (25). One-day-old rats from different litters were randomly assigned to either the experimental malnourished group or the control group. In the control group, pups were fed by nursing dams that suckled six to eight pups. In the malnourished group, nursing dams fed 16 pups. Pups were weighed from the first day until weaning (from 1 to 21 days after birth).

Well-nourished rats were selected from different control litters, and malnourished rats were selected from different experimental litters. In the experimental group, degree of malnutrition was established according to the classification used for children described by Gómez *et al.* (22). Malnutrition was categorized by weight deficit compared to weight of age-matched control rats (WN): moderate or second degree (MN2nd) when weight deficit was 25–40%; and severe or third degree (MN3rd) when weight deficit was greater than 40%. Rat pups had other physical signs of malnutrition, such as sparse hair, bone fragility and low activity levels.

Biochemical measurements of malnutrition

Total serum proteins were quantified in 16 MN3rd rats, in 11 MN2nd rats and in 16 WN control rats, using the method previously described by Lowry *et al.* (26). Serum cholesterol and triglyceride levels were measured using Reflotron Plus System (Roche Diagnostics, Basel, Switzerland).

BrdUrd labelling

Rats were injected 1 mg/g body weight, i.p., BrdUrd (5-bromo-2'-deoxyuridine; Sigma, St Louis, MO, USA). At 2, 4, 6 and 8 h after injection, animals were sacrificed by cervical dislocation and spleens were dissected; cells were obtained by sieving tissues through a nylon screen. Cells were resuspended in phosphate buffered saline solution (PBS, pH = 7.4; Microlab, Mexico City, Mexico) and were fixed in cold 70% ethanol (Gly 5 mm, pH = 2; Sigma) and stored at –20 °C for 24 h; they were then washed in PBS. BrdUrd labelling was detected using 5-bromo-2'-deoxyuridine Labelling and Detection Kit I (Roche, Mannheim, Germany). Cells were incubated in an anti-BrdUrd monoclonal antibody and nuclease, for 60 min at 37 °C and 30 min with secondary FITC-labelled goat anti-mouse monoclonal antibody. Propidium iodide (Sigma, 5 µg/ml, final concentration) was added followed by incubation for 15 min. Cells were fixed in paraformaldehyde (1% in PBS). A total of 20 000 cells were analysed by flow cytometry (FACSscan model; Becton Dickinson, San Jose, CA, USA). Contour graphs were utilized to establish percentages of BrdUrd-positive cells (Fig. 1). CellQuest Software (V 3.0.1; Becton Dickinson) was used for data acquisition and analysis.

Percentages of cells that incorporated BrdUrd were calculated from the total number of analysed cells. Time in phases G₁ (T_{G1}), S (T_S), G₂ (T_{G2}) and total cycle time (T_C) were obtained from incorporation data after 8 h based on the method proposed by Eidukevicius *et al.* (27). Total number of cells labelled in S-phase (S^l) during a pulse can be estimated at any time interval shorter than duration of the cell cycle as follows:

$$S^l = S^{lu} + G_2^{lu} + 1/2G_1^{ld} + 1/2S^{ld}.$$

Total number of dividing cells labelled in G₁ (G_1^{ld}), in S-phase (S^{ld}); non-divided cells in S-phase (S^{lu}) and non-divided cells in G₂ were analysed using contour graphs and histograms of positive cells. Equations used to establish timing of each phase of the cycle, T_C , growth fraction (GF), labelling index of proliferating cells (LI_p) and labelling index (LI) are shown in Table 1.

Figure 1. (a) Dot plot of IP-W versus IP-A parameters (DNA content) to select population of spleen cells, discriminating doublets. (b) Histogram of DNA content versus cell frequency where peaks correspond to cells in G₁, S and G₂/M phases. (c) Contour plot indicating cells in different phases of the cell cycle and cells that incorporated BrdUrd during the process of DNA synthesis. Figure is of cells from WN rat pups.

Table 1. Equations to establish the timing of each phase of the cycle

Cell cycle parameter	Equations
S^l	$S^{lu} + G_2^{lu} + \frac{1}{2}G_1^{ld} + \frac{1}{2}S^{ld}$
T_S	$(S^l / (G_2^{lu} + \frac{1}{2}(G_1^{ld} + S^{ld}))) \times t$
T_{G2}	$(\frac{1}{2}(G_1^{ld} + S^{ld}) / S^l) \times t$
T_{G1}	$t \times T_{G2} - (\frac{1}{2}S^{ld} / S^l) \times T_S$
T_C	$T_{G1} + T_S + T_{G2}$
GF (%)	$(LI / LI_P) \times 100$
LI_P	$(T_S / T_C) \times T_S$
LI (%)	$(S^l / n_t - \frac{1}{2}(G_1^{ld} + S^{ld}) - (G_2 - G_2^{lu})) \times 100$

G_1^{ld} , labelled divided G₁ cells; G_2^{lu} , labelled undivided G₂ cells; GF, growth fraction; LI, labelling index of the whole population; LI_P , labelling index of proliferating cells; n_t , total number of BrdUrd positive and negative cells per sample; S^l , labelled S cells; S^{lu} , labelled undivided S cells; t , time of BrdUrd incorporation; T , time of phase cycle.

Statistical analysis

Body weight and weight of spleens, numbers of cells, weight deficits and biochemical measurements are expressed as mean $\pm$ standard deviation. BrdUrd incorporation results are expressed as mean $\pm$ standard error of four rats by incorporation time. Statistics were calculated using BioEstat 5 software (Manuel Ayres, Universidade Federal do Pará, Pará, Brasil). All groups were compared using the non-parametric Kruskal–Wallis and Dunnett's tests. Level of significance was set at $P < 0.05$; at least 15 000 cells/sample were analysed.

Results

Table 2 shows average weight of body and spleen and standard deviation of control rat pups and malnourished animals (MN) with second and third degree severities. 21 days after birth, observed weight deficits of MN2nd and MN3rd rats compared to WN group were 34.5%

Table 2. Body and spleen weights of well-nourished and malnourished rat pups

Variable	WN	MN2 nd	MN3 rd
Body weight (g)	54.4 ± 2.3	36.2 ± 2.0	26.2 ± 3.3*
Body weight deficit	—	34.5 ± 3.7	51.9 ± 6.0
Spleen weight (mg)	308.7 ± 71.9	154.7 ± 40.0	117.1 ± 43.4
Spleen weight deficit (%)	—	49.9 ± 13.0	61.7 ± 14.2
Spleen weight (mg)/body weight (g)	5.7 ± 1.4	4.3 ± 1.0*	4.4 ± 1.4*
Cell number (×10 ⁶)/spleen	196.4 ± 49.4	113.7 ± 34.8*	79.7 ± 39.3***
Cell number/spleen deficit	—	45.7 ± 22.4	62.9 ± 13.8
Cell number (×10 ⁶)/100 mg spleen	73.5 ± 17.1	78.2 ± 28.8	68.1 ± 22.4
Total serum proteins (mg/ml)	57.1 ± 15.5	40.1 ± 2.2	27.9 ± 10.4*
Serum triglycerides (mg/dl)	272.6 ± 22.5	165.4 ± 22.6	175.3 ± 20.0*
Serum cholesterol (mg/dl)	129.0 ± 5.9	108.1 ± 3.0	115.0 ± 5.9*

Values are the mean ± standard deviation. *Significant differences in the WN group, $P < 0.05$; **Significant differences MN2nd versus MN3rd, $P < 0.05$. WN, $n = 16$; MN2nd, $n = 11$; MN3rd, $n = 16$. The cholesterol serum concentration was <100 mg/dl in ten of the MN3rd rats.

and 51.9%, respectively. Significantly lower cholesterol and triglyceride levels were observed in serum of both groups of malnourished rats ($P < 0.05$).

Weights of spleens were lower by approximately 44% in MN2nd group and by 63% in MN3rd group. This observation was corroborated by the cell number analysis as cellularity in malnourished animals was 43% and 52% lower compared to the WN group. Ratios between spleen and body weights were clearly lower in both malnourished groups compared to the control group ($P < 0.05$ for both). However, statistically significant differences were not observed between numbers of cells and spleen-to-weight ratios. This result indicates that cell density was maintained in spleens of malnourished and well-nourished rats, although sizes of spleens was less.

Percentages of cells in different phases of the cell cycle were analysed using DNA content as marker, based on fluorescence of propidium iodide (Fig. 1a and 1b). Figure 2 shows cell distribution in each phase of the cycle. In all phases, significant differences in numbers of cells were observed, between malnourished rats

Figure 2. Distribution of cells in each phase of the cell cycle. Data for each group are shown as average ± standard error (SE). (a) WN ($n = 9$) rat spleen cells. (b) MN2nd ($n = 8$) rat spleen cells. (c) MN3rd ($n = 10$) rat spleen cells. Both malnourished groups had significantly lower percentage of cells in S-phase and G₂/M compared to well-nourished rats ($P < 0.05$).

compared to WN pups (Fig. 2) ($P < 0.05$). BrdUrd incorporation increased over time in well-nourished rats, indicating active cell proliferation. However, cells of malnourished animals had lower rates of BrdUrd incorporation after 2 h compared to WN cells, reflecting reduced proliferation (Fig. 3).

Figure 4 shows distribution of BrdUrd-positive cells in each of the cell cycle phases. In both malnourished groups, percentages of BrdUrd-positive cells was lower in each phase of the cycle. In MN2nd animals, change in distribution of cells was observed after 8 h, with increase in percentages of positive cells in S-phase and reduction in G₁. Table 3 shows labelling index and duration of each of the phases of the cell cycle. T_{G1} was

lower in the MN2nd group ($3.4 \text{ h} \pm 0.3$) compared to WN group ($5.4 \text{ h} \pm 0.5$), whereas T_{G1} in MN3rd group ($5.5 \text{ h} \pm 0.4$) was similar to the WN group. T_S was

Figure 3. Percentage of BrdUrd+ cells *in vivo* in spleen of well-nourished (WN) rats and moderately (MN2nd) and severely (MN3rd) malnourished pups after 2, 4, 6 and 8 h. Results indicate minor incorporation of BrdUrd in both groups of malnourished rats. ($P < 0.05$).

significantly higher in MN2nd group ($9.6 \text{ h} \pm 1.1$) compared to WN group ($3.6 \text{ h} \pm 1.1$), and non-significant increase was observed in the MN3rd group ($4.3 \text{ h} \pm 1.1$). The T_{G2} was similar for all the groups (WN $1.4 \text{ h} \pm 0.3$, MN2nd $1.4 \text{ h} \pm 0.1$ and MN3rd $1.2 \text{ h} \pm 1.1$). T_C was significantly higher in MN2nd group, and increase was also observed in MN3rd group, although it did not reach significance. GF was significantly lower in the MN3rd group ($19.9 \pm 1.3\%$) compared to MN2nd and WN groups ($30.9 \pm 1.8\%$ and $47.0 \pm 4.4\%$, respectively).

Discussion

This study has evaluated effects of malnutrition on *in vivo* proliferation of rat pup spleen cells during lactation, representing time of accelerated growth when effects of malnutrition are more significant than in later stages. Malnutrition at lower age has been shown to create permanent effects (28).

Figure 4. Distribution of cells in each phase of the cell cycle that incorporated BrdUrd *in vivo* in well-nourished rats (WN) and in moderately (MN2nd) and severely (MN3rd) malnourished pups at 2, 4, 6 and 8 h. Results indicate minor incorporation of BrdUrd in both groups of malnourished rats, $P < 0.05$.

Table 3. Estimated duration of the cell cycle phases in the spleen cells of well-nourished and malnourished rats using the Eidukevicius method after 8 h of a BrdUrd pulse

	T_{G1}	T_S	T_{G2}	T_C	GF (%)
WN	5.4 ± 0.5	3.6 ± 1.1	1.4 ± 0.3	10.5 ± 0.7	47.0 ± 4.4
MN2 nd	$3.4 \pm 0.3^{***}$	$9.6 \pm 1.1^{***}$	1.4 ± 0.1	$14.4 \pm 0.9^{***}$	$30.9 \pm 1.8^*$
MN3 rd	5.5 ± 0.4	4.3 ± 1.1	1.2 ± 1.1	11.0 ± 0.7	$19.9 \pm 1.3^*$

Values are the mean $\pm$ standard error. Times are given in hours. Different superscript letters are statistically significant; *significant differences in the well-nourished (WN) group, $P < 0.05$; **significant differences in the moderately (MN2nd) and severely (MN3rd) malnourished rats, $P < 0.05$.

Malnutrition reduced spleen-to-body weight ratio in both of malnourished rat pup groups compared to healthy control animals. Numbers of cells in spleens were lower in malnourished animals, but values of cell density remained very similar among the three groups. This result indicates that cell numbers determines weight of the spleen. However, other studies have reported that proportions of cell subpopulations in the spleen are altered as a result of malnutrition, with reduced number of T lymphocytes and higher numbers of non-lymphoid cells (19). Results of this study show changes in cell proliferation with different effects depending on degree of malnutrition. Proportions of cells in the various phases of the cell cycle were reduced in both groups of malnourished rats. These data provide evidence that the cell cycle was altered as a result of malnutrition. *In vivo* BrdUrd incorporation enables evaluation of proliferation kinetics and effects induced by malnutrition (21).

The method of Eidukevicius *et al.* (27) was selected to establish cell kinetics as it allows calculation of periods of time cells are in G_1 , S and G_2 phases. The assumption is that the method used can estimate total number of cells labelled in S phase during BrdUrd after any time interval shorter than duration of the cell cycle. The method described by Begg *et al.* (29) and cubic method (30) were also tested to calculate T_{G_2+M} , T_s and T_{pot} . However, the main disadvantage of these two methods is that it is impossible to calculate lengths of time of all phases using the same method. In addition, it was not possible to obtain reliable results using data from all of samples (results not shown). An advantage of the selected method is the option to calculate growth fraction (GF%), valuable in this study. Time spent in M-phase is absent in the equation proposed by Eidukevicius *et al.* (27) as the technique used to isolate nuclei does not allow cells to be captured in mitosis. The impact of this omission on results of the study should be negligible, regarding estimation of total number of labelled cells.

BrdUrd incorporation results demonstrate that there were fewer proliferating cells in both malnourished groups of pups and that cells from those with severe malnutrition had lowest BrdUrd incorporation out of all three of groups. Alterations in duration of phases were observed in the moderately malnourished group; T_{G_1} was diminished and T_s and T_C were increased compared to WN rats. Results from rats in the severe malnutrition group indicated that the effect was produced primarily by an important reduction in fraction of growing cells (GF%), with non-significant increase in T_s and T_C . Winick and Noble (28) reported that malnutrition during lactation affects spleen growth due to reduced cell division, and that this decrease was observed even after

nutritional recovery up until 133 days of age. Our results confirm that severe malnutrition diminished spleen cell proliferation in rats, due to differences in competence during lactation (from birth to 21 days).

Further studies have shown that malnutrition affects different phases of the cell cycle in different types of tissues. Rose *et al.* (31) showed that cells of intestinal mucosa have prolonged S-phase in rats on a restricted protein diet, whereas delay in the G_2 and S-phases were observed in hair follicles and epidermis of rats malnourished from birth (32). Spermatogonia and Sertoli cells of rats with caloric deficiencies during lactation have been observed to have delayed G_1 and S-phases (33). Proliferation of cells in the bone marrow of malnourished rats is slower than in cells of well-nourished rats and reduced percentage of proliferating cells has also been observed (20–22). Gomez *et al.* (22) showed that the cell cycle is prolonged in malnourished rats. $G_1 + 1/2M$ phases are more sensitive to malnutrition, whereas no differences have been found in S and $G_2 + 1/2M$ phases. It has been proposed that malnutrition makes it difficult for cells to complete G_1 , which could be related to deficiency of essential nutrients required for synthesis of proteins necessary for activities specific to G_1 , and to subsequent progression to S-phase (34). In malnourished mice, numbers of progenitor and $CD5^+$ cells from bone marrow, in G_0 increased, with reduction in number of cycling cells (35,36).

Studies evaluating DNA damage in lymphoid tissue cells in malnourished rats have reported that malnutrition is associated with increased DNA damage in the spleen, bone marrow and blood lymphocytes, which could be related to lower capacity to repair damage (37). Evaluation of micronuclei in cells of malnourished children has shown that DNA damage in children with moderate malnutrition is lower than DNA damage in children with severe malnutrition, and may be related to availability of micronutrients and levels of oxidative stress (24). The cell cycle has checkpoints that evaluate DNA integrity. If the cell is damaged, cycle cannot progress (38–41). It is possible that levels of DNA damage would have different effects on the cell cycle. Thus, additional studies are required to evaluate relation of levels of micronutrients and oxidative stress with levels of DNA damage and the cell cycle. Length of the cell cycle was higher in cells of rat pups with moderate malnutrition, as more time was needed to complete S-phase, probably related to the increased DNA damage and induction of the repair mechanisms. Severe malnutrition reduces numbers of proliferating cells and restricts initiation of the cell cycle to a small fraction of the cell population, probably related to deficiency of nutrients and increased levels of DNA damage.

In conclusion, moderate malnutrition increased time required for DNA synthesis and time of the total cycle, and severe malnutrition reduced growth fraction of spleen cells in malnourished rats during lactation.

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