Cytogenetic Studies in Bone Marrow Cells From Wistar Rats in Protein Malnutrition

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The effect of severe protein deficiency at weaning has been studied in bone marrow, which is a primary lymphoid organ. Our experimental model of secondary immunodeficiency in Wistar rats has shown: (1) a decreased number of viable bone marrow cells ($P < .0001$); (2) diminished percentage of mitosis ($P < .01$); and (3) severe alteration in the percentage of chromosome pairs 3, 11, and 12 bearing nucleolar organizer regions (NORs) ($P < .05$). This last finding indicates a poor ribosomal gene activity. These alterations were reverted after the oral administration of a 20% casein diet during 5 to 9 days. However, there were no karyotype variations between the experimental groups. We conclude from these results that severe protein deficiency at weaning alters several aspects of bone marrow cell proliferation and ribosomal gene activity as determined by the number of silver stained nucleolus organizer regions.

IT IS KNOWN THAT immunologic functions and immunoregulatory mechanisms are influenced by nutritional factors.[1] The kinetics of cellular proliferation in lymphoid organs suggest that these tissues are susceptible to the effects of protein deficiency. It has been shown in experimental models that severe protein deficiency seems to have a great impact on the thymus and other lymphoid organs, which are atrophied and depleted of lymphocytes.[2,3] Malnutrition, and specially protein deficiency, provokes an impairment of cell-mediated immunity, immune responses, and secretory antibody responses.[4]

Results from our laboratory in a model of secondary immunodeficiency due to severe protein deprivation at weaning have shown that the thymus is rendered alymphoid[5,6] accompanied by the presence of a pre–B-cell population in the Peyer's patches.[7] Small intestines presented a third of the T-cell numbers found in 39-day-old aged-matched control rat lamina propria for all subsets studied. Moreover, a lack of immunoglobulin (Ig)A and IgG B cells and a third of IgM B cells were observed in the gut lamina propria.[8]

The possible role of nutritional deficiencies in affecting cellular genetic integrity has been well studied. Cells from malnourished rats showed reduced DNA, RNA, and protein content,[9] as well as low values of RNA polymerase.[10] In addition, cell cycle changes have been documented.[11]

In a high turnover tissue, such as bone marrow, deficiency in nutrients causes an elongation of the generation time determined by differential staining of sister chromatides in vivo,[12] as well as in vitro.[13]

Studies on chromosomal aberration in protein energy malnourished children and animals suggested both positive and negative effects. Armendares et al[14] and Betancourt et al[15] observed higher aberration rates in severely malnourished infants as compared with normal children. Thorburn et al[16] did not find significant differences between normal children and children suffering from protein calorie malnutrition. Khouri and McLare[17] also could not find any correlation between the degree of malnutrition and the incidence of chromosomal lesions. However, results in experimental animals suggested that malnutrition per se has deleterious effects on chromosomes.[18] It has been reported in rats that a low-protein diet has an adverse influence on the structural integrity of bone marrow chromosomes.[19,20]

The above reports, however, consist of cytogenetic studies in which only chromosome aberrations were analyzed. In none of them is it mentioned how malnutrition affects the number of viable bone marrow cells or the number of mitoses. Moreover, what we consider most important is that there are no previous studies in animal models regarding how protein deprivation affects the number and distribution of nucleolar organizer regions (NORs) (indicators of ribosomal gene activity).

NORs, the sites of ribosomal RNA (rRNA) gene clusters on particular chromosomes can be selectively stained by the silver staining method.[21] The number and distribution of Ag-stained NORs (Ag-NORs) differs between species.[21,22] In Wistar rats, the NORs are on secondary constrictions on the short arms of chromosomes 3 and 12 and on the telomere of the short arm of chromosome 11.[23] It has been established that the Ag-NOR proteins are indicators of cell proliferation and ribosomal gene activity since only sites active in rRNA transcription prior to division would be stained by this technique; inactive sites would not be stained.[24] NORs vary in size and shape according to nucleolar transcription. Therefore, it is likely that sizes and numbers of Ag-NORs are dependent on the proliferative status of the cell.

Severe protein deprivation at weaning in our experimental model (Wistar rats) resembles protein malnutrition in children. Therefore, this study was performed to investigate the effect of a protein-free (PF) diet on: (1) chromosome integrity and (2) to analyze the frequency of chromosome pairs 3, 11, and 12 expressing Ag-NORs, these being important indicators of ribosomal gene activity.

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MATERIAL AND METHODS

Animals

Rats of the Wistar strain (close colony from the animal facilities of the School of Pharmacy and Biochemistry, Buenos Aires, Argentina) of either sex were used.

A total of 8 litters were used (litter size, 8 animals per dam). Weanling animals were randomly divided into the different groups. Experiments were performed with 5 to 7 animals per group.

All animals were housed individually in screen bottom cages and were exposed to a 12-hour light–darkness cycle; room temperature was kept at 21°C ± 1.0°C; water and diets were offered ad libitum.

The weights of all the animals were recorded at weaning (selecting those weanling rats with ± 40 g) and thereafter until they were killed.

Weanling rats aged from 21 to 23 days were protein-depleted by being fed a protein-free (PF) diet, and animals that had lost 25% of their initial body weight (PF) were selected.

A protein diet containing casein as the only source of protein (20% casein) was given to depleted rats for 5 days (PF group + 5 days 20% casein: R5 group), 9 days (PF group + 9 days 20% casein: R9 group), and 21 days (PF group + 21 days 20% casein: R21 group).

Well-nourished control groups of 39, 45, and 60 days of age (C39, C45, and C60) received commercial laboratory diet (Cargill, Buenos Aires, Argentina, 24.6% protein). The commercial diet contains proteins from animal and vegetable origin with biologic value 70 to 75. This project has been approved by the University of Buenos Aires Ethical Committee.

Experimental Diets

Experimental isocaloric diets were prepared as previously reported[25]; to a basal concentrated diet, containing all the essential nutrients except protein, casein was incorporated in the necessary amount to provide 20% protein and then filled up to 100 g by adding dextrin. Casein is a protein with high biologic value (BV, 80 to 85); therefore, it is not supplemented with limiting amino acids. In the PF diet, casein was omitted and replaced by dextrin. Therefore, the calorie density of the diets is the same. This repletion diet is nutritionally adequate, and it has been used since the beginning of our work line. Components of the experimental diets are as follows. PF diet (g/100 g): casein (as calcium caseinate, ≥ 85% protein), 0.00; mineral mix, 5.00; vitamin mix, 0.25; colin (as sodium chloride), 0.15; corn oil, 5.00; dextrin, 89.6; total amount of protein, 0%. Casein diet (g/100 g): casein, 23.13; mineral mix, 5.00; vitamin mix 0.25; colin, 0.15; corn oil, 5.00; dextrin, 66.47; and total amount of protein, 20%. Mineral mix (g/100 g): CO_3Ca, 29.29; PO_4HCa2H_2O, 0.43; PO_4H_2K, 34.31; ClNa, 25.06; SO_4Mg7H_2O, 9.98; $Fe(C_6H_5O_7)6H_2O$, 0.623; SO_4Cu5H_2O, 0.156; SO_4MnH_2O, 0.121; Cl_2Zn, 0.02; IK, 0.0005; $Mo_4O_{24}(NH_4)_64H_2O$, 0.0025. Hydrosoluble vitamin mix (g/100 g): vitamin B_1, 0.50 mg; vitamin B_2, 0.50 mg; niacin, 2.50 mg; calcium pantothenate, 2.00 mg; vitamin B_6 HCL, 0.25 mg; folic acid, 0.02 mg; biotin, 0.01 mg; vitamin B_{12}, 0.002 mg; inositol, 10.0 mg; vitamin C, 5.00 mg. Liposoluble vitamin mix (g/100 g): vitamin A, 400 UI; vitamin D, 40 UI; vitamin E, 10 UI.

Chromosome Preparations

Bone marrow (BM) from femora was aspirated into RPMI 1640 medium supplemented with fetal calf serum 10%. Cells were pretreated with colchicine, 40 mg% for 30 minutes at 37°C, then treated with potassium chloride, 0.075 mol/L (Sigma, St Louis, MO) at 37°C, and fixed in methanol:acetic acid mixture (3:1). Air-dried slides were made and stained with Giemsa. All slides were coded, and for each rat, 50 well-spread cells in metaphase were selected at random and examined for aberrations. Cell viability was determined by trypan blue exclusion (0.05% saline) using an hemocytometer counting chamber.[26]

Silver Staining Method

Staining of NORs was performed by incubating slides at 37°C for 2 to 20 hours with a few drops of 50% NO_3Ag (wt/vol) solution under a coverslip. Incubation was performed in a wet chamber and in the dark. After this treatment, slides were rinsed and stained for a few minutes in 4% Giemsa.

About 100 to 150 metaphase spreads per group were randomly scored by 2 blinded investigators for the presence or absence of any visible Ag-NOR in any copy of chromosomes 3, 11, and 12. These 3 pairs of chromosomes are morphologically identifiable under the microscope.

Statistical Analysis

Results were analyzed using analysis of variance (ANOVA) followed by Tukey-Kramer test. When needed, the 2-tailed *t* test was determined. The InStat (Graph Pad Software, San Diego, CA) computer program was used.

RESULTS

Table 1 shows data of the average body weight and the number of BM cells from control and experimental groups. Both the body weight and number of BM cells are significantly decreased in rats fed a PF diet (PF group) when compared with its control (C39) ($P < .0001$). After refeeding with 20% casein diet for 9 days (R9 group), the cell number is recovered. However, body weight values, even after refeeding during 21 days with a 20% casein diet (R21), did not reach control values (C60) ($P < .001$).

The mitotic index is also significantly lower in malnourished rats (PF) when compared with their age-matched control (C39) ($P < .0005$) (Table 2). Casein refeeding during 5 days (R5) restores such situation, as the percentage of metaphases in the BM of refed animals is similar to the percentage found in their respective controls (Table 2).

To determine the percentage of positive Ag-NOR chromosomes in control and experimental animals, 100 to 150 cells in metaphase were counted per group (Table 3). In the PF group, we have observed that positive Ag-NORs in chromosomes 3, 11, and 12 are significantly decreased both in size and number

Table 1. Body Weight and Number of BM Cells in Malnourished Rats (PF) After Protein Refeeding With 20% Casein Diet During 5, 9, and 21 Days (R5, R9, R21) and Control Groups (C21, C39, C45, C60)

Group	Body Weight (g) X ± SE	No. of BM Cells × 10^8/mL X ± SE
C21 (21 d)	36.90 ± 1.70	1.63 ± 1.31
C39 (39 d)	103.90 ± 5.50	3.03 ± 0.38
C45 (45 d)	111.90 ± 6.40	2.25 ± 0.33
C60 (60 d)	164.50 ± 6.90	4.09 ± 0.59
PF (34-38 d)	27.20 ± 1.60*	0.88 ± 0.07*
R5 (39-43 d)	47.60 ± 1.60*	1.35 ± 0.12†
R9 (43-47 d)	61.90 ± 1.80*	1.79 ± 0.22
R21 (55-60 d)	109.60 ± 3.10*	3.78 ± 0.79

NOTE. X ± SE: mean ± SEM. C21, weanling rats, 21 to 23 days old, n = 5 to 9 animals per group.

*Significantly different from aged-matched controls by Student's *t* test, $P < .0001$.

†Significantly different from age-matched controls by Student's *t* test, $P < .03$.

Table 2. Frequency of Metaphases in BM Cells From Control and Experimental Groups

Groups	No. of M/500 BM cells X ± SE
C21	19.25 ± 0.63
C39	17.80 ± 0.86
C45	18.75 ± 1.65
C60	17.60 ± 1.08
PF	11.2 ± 0.80*
R5	18.75 ± 0.95
R9	17.00 ± 1.16
R21	17.25 ± 0.63

NOTE. X ± SE: mean ± SEM. N = 5 animals per group.
*Tukey-Kramer: PF *v* the remaining groups, significantly different $P < .01$.

when compared with the rest of the studied groups (Fig 1). Rats refed with a 20% casein diet during 5 days (R5) rendered the same values found in age-matched controls (Table 3). In Fig 2, a metaphase of a control animal (C60) stained by the silver staining method is shown. Chromosomes 3, 11, and 12 bearing Ag-NOR are indicated with arrows.

A total of 400 metaphases in the PF group, 350 metaphases in C60, and 350 in the R21 group was analyzed for chromosome abnormalities. Except for a fracture in the long arm of chromosome no. 1 in a metaphase of a PF animal, no chromosomal aberrations were detected in any group. We cannot conclude from our results that a PF diet affects chromosome integrity.

DISCUSSION

It is well known that malnutrition provokes diverse effects on BM cells.[11,14,18-20,27-29] It has been described that the precursors of IgA plasma cells are BM-derived cells that can be recognized in the Peyer's patches.[30] We have demonstrated in Wistar rats that protein deprivation at weaning provokes a breakdown in B-cell differentiation, which is recognizable at the level of Peyer's patches, due to the finding of immature B cells (pre–B-cell population) identified as $c\mu + s\mu$- $Thy1^+$ cells.[31]

This pre–B-cell population found in Peyer's patches of pro-

Table 3. Percentage of Ag-NORs in Chromosomes 3, 11, and 12 From Control and Experimental Groups

Groups	Total No. of Metaphases Analyzed	Percentage of Ag-Positive Chromosomes X ± SE		
		Pair 3	Pair 11	Pair 12
C21 (21 d)	167	64.48 ± 3.81	55.70 ± 7.31	48.47 ± 7.50
C39 (39 d)	114	52.85 ± 6.74	55.51 ± 3.98	50.67 ± 3.40
C45 (45 d)	127	46.56 ± 1.78	59.37 ± 5.64	51.47 ± 5.20
C60 (60 d)	148	60.16 ± 5.23	56.33 ± 4.30	44.94 ± 5.07
PF (34-38 d)	143	25.55 ± 5.21*	20.25 ± 4.26*	22.11 ± 5.73*
R5 (39-43 d)	117	62.14 ± 1.23	58.15 ± 6.50	52.14 ± 4.34
R9 (43-47 d)	152	49.89 ± 7.29	39.95 ± 2.65	40.43 ± 5.48
R21 (55-60 d)	116	60.74 ± 6.43	61.76 ± 8.00	51.20 ± 5.00

NOTE. X ± SE: mean ± SEM. N = 5 to 7 animals per group.
*Tukey-Kramer: PF *v* the remaining groups, significantly different $P < .05$.

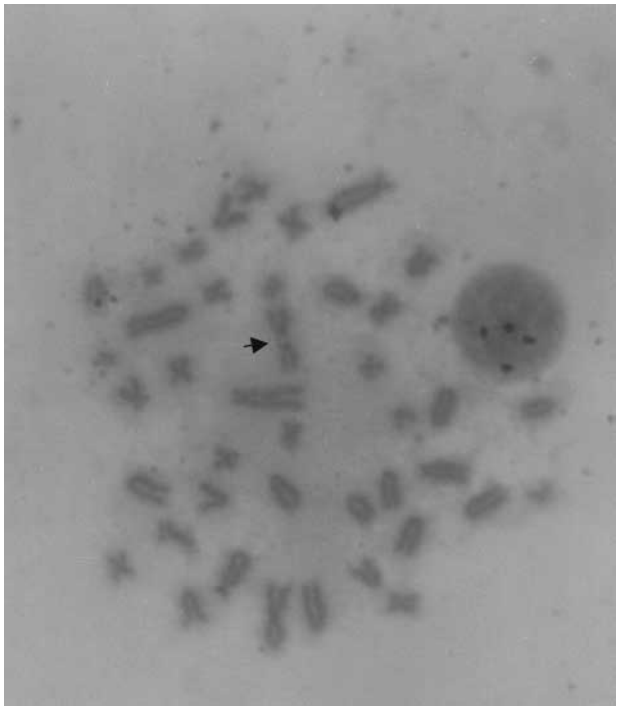

Fig 1. Silver-stained metaphase plate of a PF animal. Association between chromosomes 11 is indicated (1,000×).

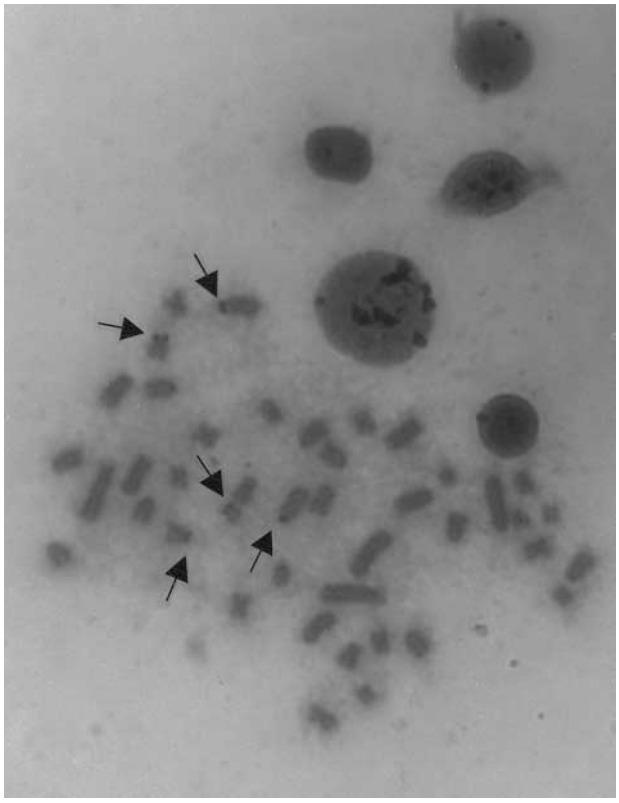

Fig 2. Silver-stained metaphase chromosomes of a control rat BM cell. The nucleolar chromosomes 3, 11, and 12 are indicated by arrows (1,000×).

tein-deprived rats (PF group) represents a BM-derived cell population that in the above conditions could not complete its differentiation.[7] We also observed in this experimental model of immunodeficiency the existence of impairment in the T-cell pathway in the thymus.[5,6] These findings led us to investigate the effect of protein deficiency on the structural integrity of BM chromosomes.

We observed that the administration of a PF diet to weaning rats provokes a significant decrease in the number of BM cells and alterations in the mitotic index, which is much lower in malnourished animals. Both the number of BM cells and the percentage of metaphases reach control values after refeeding with a 20% casein diet during 5 to 9 days. Therefore, we conclude that the PF diet is the cause of the above findings. When refeeding takes place for 5 to 9 days with a 20% casein diet, essential amino acids are provided enabling the renewal of cellular proliferation in the BM.

Although several studies have been performed to determine the effect of malnutrition over chromosome integrity, results are contradictory. Based on works determining how protein deprivation affects cellular metabolism,[11,19,20,27,28,32,33] it has been proposed that under these conditions, chromosomes may mutate. Moreover, several investigators[19,20,34] reported that malnutrition per se provokes karyotype anomalies. Meanwhile, other investigators postulate the opposite.[16,17] In the present work, we did not observe chromosome aberrations in malnourished animals.

Ag-NORs analysis determined that the percentages of NORs in chromosome pairs 3, 11, and 12 were significantly decreased in those animals receiving a PF diet in comparison to controls. Once again, the administration of a 20% casein diet during 5 days (R5) reverted this situation. This fast recovery in the R5 group may be due to an acceleration of the DNA synthesis and cell proliferation when animals are exposed to a complete nutritional diet.[35]

The evaluation of active NORs in karyotypes during mitosis is based on the presence of proteins of the ribosomal gene transcription machinery; the activity of ribosomal genes can be detected by this analysis. The present work ascertains that rRNA transcription is affected by severe protein deficiency at weaning.

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