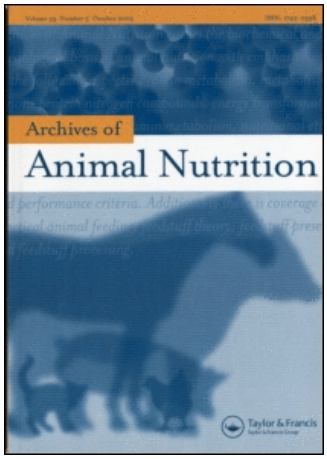

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Effects of maternal vitamin B_{12} deficiency from end of gestation to weaning on the growth and haematological and immunological parameters in mouse dams and offspring

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Vitamin B_{12}-deficiency may induce specific symptoms as neurological alterations and unspecific symptoms such as anaemia and growth retardation. In this study, maternal vitamin B_{12} deficiency from end of gestation to weaning was evaluated in mouse dams, which was provoked by feeding a vitamin B_{12}-deficient diet. The animals were divided into two groups (control and deficient). The control group received the vitamin B_{12}-deficient diet supplemented with commercial vitamin B_{12}. Compared to the control, the vitamin B_{12}-deficient dams and their offspring showed a significant decrease of body weight (by 20 and 39%, respectively), serum vitamin B_{12} concentration (by 61 and 67%, respectively), haematological values as haematocrit (25 and 26%, respectively), and IgA producer cells (by 36 and 54%, respectively). In both, vitamin B_{12}-deficient mouse dams and their offspring, histological alterations of small intestine were observed, whereas growth retardation occurred in the offspring only. This experimental murine model allows assessing the incidence of maternal cobalamin deficiency in offspring and would be useful for evaluating novel adjuncts such as functional foods to prevent vitamin B_{12} deficiency.

Keywords: vitamin B_{12}; deficiency; mice; progeny; anaemia

1. Introduction

Vitamin B_{12} (deoxyadenosyl- or methyl-colbalamin) is an important water-soluble vitamin belonging to the vitamin B complex. Together with folic acid it functions as methyl donor in the synthesis of DNA and red blood cells as well as in maintaining the health of the insulation sheath (myelin sheath) that surrounds nerve cells.

Natural sources of vitamin B_{12} in human diets are restricted to foods of animal origin. Cobalamin deficiency has been reported to cause growth retardation and severe haematological and neurological abnormalities (Toyoshima et al. 1996; Stabler 2000). This deficiency may be due to different causes: inflammation of stomach lining, congenital loss of the stomach intrinsic factor, medications that reduce stomach acid, *Helicobacter pylori* infection, a reduced consumption of food originating from animals, e.g. strict vegetarian diets (Forrellat et al. 1999), malnutrition, physiological states (pregnancy and old age). Recently, Koebnick et al. (2004) reported that pregnant women consuming a predominantly vegetarian diet over a long term have an increased

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risk of vitamin B_{12}-deficiency. Similar hazard are encountered in case of undernutrition or severe unbalanced diets. It is reported that vitamin B_{12}-deficiency in the mother's diet during the gestational period causes severe retardation of myelination of the baby's nervous system and brain atrophy (Lovblad et al. 1997). In infancy, this vitamin deficiency may cause failure to thrive, irritability, anorexia, delay and regression of neurological development, hypotonic state, coma and convulsions, severe megaloblastic pancytopenia due to a delayed DNA synthesis, myelination defects (Grattan-Smith et al. 1997; Ramussen et al. 2001) and the immune system malfunctions (Sakane et al. 1982).

The aims of this work were to set up a vitamin B_{12}-deficient murine model, and to characterise the alterations in growth and in haematological and immunological parameters that take place in mouse dams and offspring after a maternal vitamin B_{12} deficiency from the end of gestation to weaning. The development of particular experimental models for a given disease is important for assessing the efficiency of foods for specific health uses.

2. Materials and methods

2.1. Animals and diet groups

Six-weeks old pregnant female BALB/C mice (14-days pregnancy calculated from the first contact with the male) obtained from the closed colony of the breeding unit kept at CERELA Institute (San Miguel de Tucumán, Argentina) were individually housed in plastic cages (20 × 30 × 15 cm) with a litter tray of 20 × 30 × 6 cm. Animals were maintained at 20 ± 2°C with a 12-h light/dark cycle.

The animals were randomly allocated to two main groups (each of five mice) as follows: vitamin B_{12}-deficient dams (DD) and control dams (CD) groups. The animals were allowed free access to the diets and water. The pregnant mouse dams of groups DD and CD were fed the experimental diets during 30 days from the middle of gestation (day 11 from mating) until weaning (day 21 after offspring's birth). With this feeding schedule no spontaneous abortions were observed in the DD dams group, which was observed in case of feeding the DD diet prior or with mating (data not shown).

The vitamin B_{12}-deficient diet used in this study was provided by Biomedical Inc/ ICN (Irvine, CA, USA) and the composition is described in Table 1. The vitamin B_{12}-sufficient diet was produced by adding 1.3 μg commercial vitamin B_{12} (Parafarm, Bs. As., Argentina) per kg vitamin B_{12}-deficient diet.

The CD mouse dams group gave birth to about ten vitamin B_{12}-suffient offspring (CO group), while the DD mouse dams group gave birth to about five vitamin B_{12}-deficient offspring (DO group). The offspring remained with their mothers until weaning and were selected randomly for further studies without considering the sex.

The mouse dams (CD and DD groups) continued receiving their experimental diets during the suckling period. The feed intake (5.2 ± 0.6 g feed/d) was similar in both groups. The offspring received maternal milk only.

The body weight of mouse dams was recorded at the beginning of the feeding up to the weaning period. The body weight of the offspring was measured at the end of weaning (21 days of age). For statistical validation all determinations on mouse dams and their offspring were performed at five and ten mice per group, respectively. Determinations in the offspring were carried out at weaning (21 days of age).

Table 1. Composition of the B_{12}-deficient diet.

Component	Contents [g/kg]	Component	Contents [g/kg]
Vitamin free casein	220.0	Vitamin A acetate (500.000 IU/g)	1.8
DL-methionine	3.0	Vitamin D_2 (855.000 IU/g)	0.125
Iodinated casein	0.5	DL-α-tocopherol acetate	22.0
L-cystine	2.0	Ascorbic acid	45.0
Sucrose	632.0	Inositol	5.0
Alphacel, non-nutritive bulk	50.0	Choline chloride	75.0
Cottonsed oil	50.0	Menadione	2.25
Choline chloride	1.0	*p*-aminobenzoic acid	5.0
Calcium carbonate	6.250	Niacin	4.25
Manganese sulphate · $5H_2O$	0.180	Riboflavin	1.0
Zinc carbonate	0.05	Pyridoxine hydrochloride	1.0
Cupric sulphate · $5H_2O$	0.025	Thiamine hydrochloride	1.0
Chromium potassium sulfate	0.022	Calcium pantothenate	3.0
Sodium fluoride	0.005	Biotin	0.02
Sodium selenite	0.001	Folic acid	0.09
Salt mixture No. 2, U.S.P.XXII	40.0		

2.2. Blood and organ collection

At the end of the trials, the mouse dams of each group and the corresponding offspring were anesthetised by intraperitoneal injection of ketamin (5%) – xylacin (2%) (2.0 ml/kg BW; 20:1 v/v) (Bayer S.A) and bled by cardiac puncture. Blood was transferred into EDTA tubes for determining haematological parameters and into plastic centrifuge tubes for determination of vitamin B_{12} by immunoassay.

Freshly obtained small intestine was removed and processed for paraffin inclusion following the Sainte-Marie technique (Sainte-Marie 1962).

2.3. Haematological determinations

Haematokrit values and number of leukocytes and red blood cells were determined by haematocytometric methods. Differential cell counts were performed by counting 100 cells in blood smears stained with May Grünwald-Giemsa. The haemoglobin concentration was determined by colorimetric assay.

For the examination of reticulocytes, equal volumes (100 µl) of blood and 1% brilliant cresol blue (BCB) were mixed and incubated at 37°C for 15 min. Blood sample smears were prepared on glass slides with 5 µl of the cell suspension. The reticulocytes were counted by a microscope (1000 × magnification) in 10 areas of the stained smears, corresponding to approximately 1000 red blood cells. Results are expressed as percent of the total red blood cells.

2.4. Determination of vitamin B_{12} in serum

The concentration of vitamin B_{12} was measured in serum samples by electrochemiluminiscence immunoassay (ECLIA) on a Roche Elecsys 2010 automatic analyser (Roche Diagnostics, Basel, Switzerland) at the Laboratory for High Complex Clinic Analysis – Quevedo S.R.L. (Tucumán, Argentina). Results are expressed as pg/ml.

2.5. Histological studies

The small intestine was removed at the end of each treatment and processed by modified Sainte-Maries's technique (1962). Briefly, tissues were fixed in 10% formalin in phosphate saline solution (PBS) during 48 h at room temperature, and then dehydrated in successive alcohols baths (40%, 50%, 70%, 96% and 100%) for 20 min for each alcohol. At the end, samples were cleared by passing through three consecutive baths of xylene for 45 min each. The tissue was embedded in paraffin at 56°C for 3 h. Sectioning was carried out as usual, and tissue sections (3–4 μm) were placed on glass slides.

2.6. Determination of IgA producer cells in the small intestine

The number of IgA-producer cells was determined on histological slices from the ileal region near Peyeŕs patches by direct immunofluorescence test (DIFT) (Vintiñi et al. 2000). The DIFT was performed by using (α-chain specific) anti-mouse IgA FITC conjugated (Sigma–Aldrich, USA). Deparaffinised histological samples were incubated with the antibody dilution (1/100) in PBS (Phosphate Buffer Sodium 0.1 M, pH 7) solution for 30 min at 37°C. Samples were then washed three times with PBS solution and examined by using a fluorescent light microscope. Results were expressed as the number of IgA-producing cells (positive: fluorescent cell) per 10 fields (magnification 100 ×). Results were the mean of three histological slices for each animal.

2.7. Statistical analysis

A Student's *t*-test was used to compare the data of vitamin B_{12} deficient groups with the control groups (mouse dams and offspring). Significant differences were considered at $p < 0.05$. The experimental data were expressed as mean ± SD. The Ethical Committee for animal care at CERELA approved all animal protocols. All assays complied with the current laws of Argentina and followed the most recent recommendations of the Federation of European Laboratory Animal Science Associations.

3. Results

3.1. Animal growth

Compared to the control animals (CD group) (34 ± 1.5 g BW), the mouse dams fed the vitamin B_{12} deficient diet (DD group) showed no weight gain (Table 2). A significant delay in growth was also observed for the new born offspring of the DO group compared to the new born offspring from the control dams, fed a vitamin B_{12}-sufficient diet (Figure 1). Likewise, at weaning the offspring coming from the vitamin B_{12} deficient dams (DO group) showed a 34% lower body weight compared with the weaned young coming from the control dams (CO group) (Table 2).

3.2. Haematological determinations

The haematological values of dams and offspring are shown in Table 2. The vitamin B_{12}-deficient dams showed a significant percentage of diminution ($p < 0.05$) in the haematokrit (25%), % reticulocytes (62%), and haemoglobin (26%) values compared to the CD group. A similar tendency was observed in the corresponding weaned offspring. No statistically significant differences in the total number of leukocytes between the

Table 2. Effect of the maternal vitamin B_{12} deficiency on body weight, haematological parameters, serum concentration of vitamin B_{12} and the number of IgA producer cells of mouse dams and offspring (means ± SD).

	Experimental groups			
	Dams		Weaned offspring	
	B_{12}-sufficient CD ($n = 5$)	B_{12}-deficient DD ($n = 5$)	B_{12}-sufficient dams CO ($n = 30$)	B_{12}-deficient dams DO ($n = 30$)
Body weight [g/animal]	34 ± 1.5	27 ± 1.2*	13 ± 1.4	8 ± 0.9*
Haematokrit [%]	50.2 ± 5.6	37.8 ± 2.6*	48.2 ± 2.5	35.8 ± 3.3*
Reticulocytes [%]	4.8 ± 0.5	2.1 ± 0.2*	4.3 ± 0.4	2.2 ± 0.2*
Haemoglobin [g/dl]	16.9 ± 2.4	12.8 ± 1.6*	11.7 ± 1.5	8.9 ± 1.3*
Vitamin B_{12} [pg/ml serum]	824 ± 59	359 ± 22*	688 ± 54	296 ± 32*
Number of IgA^+ cells/10 fields	101 ± 4.1	65 ± 2.8*	82 ± 4.2	38 ± 2.9*

*Means are significantly different to respective control groups ($p < 0.05$).

Figure 1. Newborn offspring. Offspring coming from mouse dams fed the vitamin B_{12} sufficient diet (CO) and the vitamin B_{12} deficient diet (DO).

vitamin B_{12}-deficient and -sufficient groups (dams and offspring) were observed (data not shown).

3.3. Serum vitamin B_{12} concentration and histological observations

The concentration of vitamin B_{12} in serum samples of the DD group (359 ± 22 pg/ml) and the offspring group DO (296 ± 32 pg/ml) was significant lower than in the control groups (CD and CO).

The examination of the morphological architecture of the small intestine of mouse dams and weaned young belonging to vitamin B_{12}-deficient and -sufficient groups revealed that the nutritional vitamin B_{12}-deficiency determined severe histological alterations, e.g.

leukocyte infiltrations, in both adults and young animals compared to the control groups (results not shown).

3.4. Determination of IgA producer cells in the small intestine

The number of IgA^+ cells in the small intestine of dams and offspring are shown in Table 2. Compared to the control groups (CD and CO) the number of the IgA-producing cells in the vitamin B_{12}-deficient animals (DD and DO groups, respectively) were significantly decreased by 36 and 54%.

4. Discussion

Cobalamin deficiency can produce severe symptoms, some of them are irreversible. It is well documented that animals deprived of vitamin B_{12} exhibit besides growth retardations (Ebara et al. 2001) also malfunctions in the immune system and severe haematological and neurological abnormalities (Stabler 2000). During pregnancy and lactation, micronutrient deficiencies are one of the major complications promoting infectious processes. This is due to the high nutritional requirements to support foetal and infant growth as well as maternal metabolism (Cunningham-Rundles et al. 2005). Severe growth retardation in weaned young as a result of this kind of nutritional deficiency was also reported (Nakao et al. 2006).

In the present study we described an experimental murine model, which was set up from a maternal vitamin B_{12} deprivation in mouse dams to estimate the effects of this vitamin deficit on dams and their offspring. This experimental model allowed determining the histological, haematological and immunological alterations taking place as a consequence of this deficiency.

Proteins, some minerals and vitamins, play important roles in erythropoiesis and in the survival of red blood cell. Layrisse et al. (1998) dealt specifically with the physiological requirements and recommended necessary intakes of iron, folate and vitamin B_{12} for the process of erythropoiesis. In our study, mouse dams fed a vitamin B_{12}-deficient diet and their weaned young showed a lower serum concentration of vitamin B_{12}, which support the significant decrease in haemoglobin, haematocrit and reticulocytes without morphological alterations in blood cells. Similarly, no haematological abnormalities, e.g., megaloblastic cells, under normoxic conditions in vitamin B_{12}-deficient rats were reported (Ebara et al. 2003). The reduction in the average number of reticulocytes (the young red blood cells) in vitamin B_{12}-deficient mice (mouse dams and offspring) would mean a lower production of red blood cells by the bone marrow.

The intestinal microenvironment is a complex network of interactions among the microorganisms of the resident flora, nutrients, the epithelial cells, and the immune cells associated with the gut, mainly the IgA^+ producing cells that contribute to the local homeostasis. In cases of nutritional deficiencies an impaired immune response is obtained, which affects the body's defence mechanisms, disturbs the ecological barrier and induces histological damage. In the dams and offspring described in this study, histological alterations of the gut mucosa and a significant decrease in the number of IgA^+ producing cells in the small intestine in the vitamin B_{12}-deficient animals were observed. This situation was due to the vitamin B_{12}-deficiency generated in the deficient-dams during pregnancy and suckling periods. Moreover, a decrease in IgA^+ cells together with histological damages in the small intestine was found in malnourished mice (Gauffin Cano et al. 2002).

This vitamin B_{12}-deficient murine experimental model would be a useful tool for evaluating the efficiency of functional foods containing vitamin B_{12}-producer micro-organisms to prevent the nutritional deficit of cobalamin (Taranto et al. 2003).

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