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Cellular and humoral immunity in rats after gestational zinc or magnesium deficiency

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The effects of gestational Mg or Zn deficiency on the humoral or cellular immunity of newborn rats were investigated. Mg deficiency was induced by feeding a diet containing 180 ppm Mg from day 0 to day 21 of gestation and Zn deficiency was induced by feeding a diet containing 1.5 ppm Zn from day 0 to day 19. Controls were fed a diet with 1,000 ppm Mg and 100 ppm Zn from day 0 to day 21. Thereafter, all maternal rats and newborns were fed diets with normal amounts of Mg or Zn. Three and six weeks after birth, T-cell subpopulations in blood and thymus and B-cells in blood of the newborns were detected by flow cytometry. Plasma contents of IgG, IgM, and IgA were determined by radial immunodiffusion. Mg deficiency reduced litter size and pup weight. Three weeks after birth, the total number of leukocytes and lymphocytes in blood was significantly decreased, due to a reduction of T-helper and cytotoxic T-cells. Activated T-cells and B-cells were unchanged. Six weeks after birth, T-cell subpopulations approached controls values, whereas IgG content in plasma was slightly reduced. Gestational Zn deficiency reduced litter size and induced malformations. Three and six weeks after birth, body weight, number of leukocytes, lymphocyte, and T-cell subpopulations were not significantly changed. Plasma IgM was decreased 3 weeks after birth in correlation to the number of B-cells, which represented only 4% of total lymphocytes. These effects were repaired by the sixth week. Plasma IgG was reduced at 6 weeks. No effects on T-cell subpopulations in isolated thymocytes were detected after gestational Mg or Zn deficiency. (J. Nutr. Biochem. 7:327–332, 1996.)

Keywords: zinc deficiency; magnesium deficiency; gestation; immunity; newborns; rat

Introduction

The immunocompetence of an individual is essentially dependent on the availability of a sufficient amount of trace elements and minerals. Many reports exist demonstrating diminished immune function after induction of trace element or mineral deficiencies in experimental animals, but also in humans such deficiencies have been shown to produce immune defects (reviews in [1,2]).

In this respect special attention has been paid to zinc (reviews in [3–6]). Zn deficiency has led to thymic atrophy, reduced production of thymic hormones (thymopoeitin, thymulin), and reduced cellular and humoral immune response. Zn deficiency, however, does not only affect the immune system but induces various defects such as reduced growth, parakeratoses, and others. In rodents, reduced Zn supply during fetal growth leads to malformations of the skeleton and eyes, increased rate of resorptions, and reduced survival of the newborns (as reviewed in [6]). The immunocompetence of newborns was found to be reduced even after marginal Zn deficiency.[7,8] Beach et al.[9] showed in mice that gestational Zn deficiency induced a drastic reduction of IgM in the F_1 and even in the F_2 and F_3 generations.

Similarly, injured immunity has been found in Mg deficiency (review in [10]). In Mg-deficient animals thymic atrophy,[11] reduced cellular[12] and humoral[13,14] immune response as well as induction of malignant T-cell lymphoma[15] have been reported. Gestational Mg deficiency severely affects the fetus, leading to increased number of resorptions, reduced birth weight, malformations, fetal anemia, and neonatal abnormalities.[16] In rats, gestational Mg deficit reduced thymus weight and plaque-forming reaction to sheep eryth-

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rocytes in the newborns.[17] No data are available that further analyze cellular immunity in the offspring after gestational Zn or Mg deficiency with regard to effects on lymphocyte subpopulations. During the last few years monoclonal antibodies were developed to characterize subsets of lymphocytes in rats. We, therefore, investigated cellular immunity of 3- and 6-week old rats which had been subjected to gestational Zn or Mg deficiency by measuring T-cell subpopulations and B-cells by fluorescence antigen coupled flow cytometry (FACS) in peripheral blood and in the thymus; additionally, humoral immunity was determined by measuring IgG, IgM, and IgA in plasma by radial immunodiffusion. Mineral deficiencies were only induced prenatally to distinguish between those effects that might be produced by Mg or Zn deficiency during postnatal development.

Methods and materials

Virgin female Wistar rats (Institut für Embryopharmakologie, Freie Universität Berlin) weighing about 200 g, were kept on a 12-hr light/dark cycle. The rats were mated from 8 to 10 a.m., and impregnated females were identified by the presence of copulatory plugs. This day was designated as day 0 of gestation. A total of 32 impregnated rats was used.

After randomization, the rats were divided into three experimental groups: control, 9 rats; Mg deficiency, 9 rats; Zn deficiency, 14 rats.

The rats were fed ad libitum with a commercial diet (Ssniff, Soest, FRG) consisting of 24% casein, 50% starch, 11% glucose, 3% soybean oil, 5% cellulose powder, and 1% vitamin mix. One kg diet contained 18 g $CaCO_3$, 9.75 g $Ca_5(PO_4)_3(OH)$, 0.0005 g $CoCl_2$, 0.025 g $CuSO_4$, 0.5 g Fe-(II)-fumarat, 11 g K_2HPO_4, 0.0005 g KI, 0.45 *g* $MnSO_4$, 7.6 g NaCl, 0.0095 *g* NaF, 7.1 g Na_2HPO_4, 0.0005 g Na_2MoO_4, 0.019 g $NH_4Al(SO_4)_2$ (without Mg and Zn salts). Zn in casein was removed by washing twice with 1% EDTA and subsequently with bidistilled water before mixing the diet. The mixed basal diet contained 75 ppm Mg and 1.5 ppm Zn (measured by atomic absorption spectrophotometry after ashing with conc. HNO_3). By adding the respective amounts of $MgCl_2$ and $ZnCl_2$ to the basal diet the following contents of Mg and Zn were achieved as verified by atomic absorption spectrophotometry: control diet, 1,000 ppm Mg, 100 ppm Zn; Mg-deficient diet, 180 ppm Mg, 100 ppm Zn; Zn-deficient diet, 1,000 ppm Mg, 1.5 ppm Zn.

The Mg content of the diet was set to 180 ppm because lower Mg contents in the diet induced a high fetal mortality.[18] The control and Mg-deficient diets were fed until day 21 of gestation, the Zn-deficient diet was fed until day 19 only, as after this day Zn-deficient dams reduced their food intake and surviving newborns were not obtained.[18] However, when Zn-deficient pregnant rats were nutritionally rehabilitated within the last 3 days of pregnancy, they delivered and nursed their pups normally.[19] After day 21 or day 19, all rats were fed a commercial rat diet (Ssniff, Soest, FRG). All animals received bidistilled water ad libitum.

After birth, the litter size was adjusted to eight. Newborns were left with their mothers until day 21 and then kept in groups of three to five animals. Three and six weeks after birth, animals of each group were anesthetized by i.p. injection of 50 mg/kg Nembutal. Blood was obtained by heart puncture with an Na-EDTA containing syringe, and the thymus was removed and washed in phosphate-buffered saline (PBS).

Blood was centrifuged for 10 min at 1,000 g, plasma was withdrawn and stored at −30°C. Blood cells were resuspended in PBS for further measurements. Thymocytes were isolated by gently sieving the thymus into PBS and washing twice. By this procedure, more than 90% of viable cells were obtained as judged by trypan blue exclusion.

The total number of blood leukocytes was determined with a coulter counter, and the percentage of lymphocytes was counted in a blood smear stained according to Pappenheim. Lymphocyte subpopulations were determined by FACS.[20] For this purpose fluorescein isothiocyanate (FITC)- or R-phycoerythrin (RPE)-coupled monoclonal antibodies were used. Briefly, blood cells or thymocytes were incubated with a suitable dilution of monoclonal antibody solution and subsequently incubated for 30 min on ice. After lysis of erythrocytes leukocytes of blood samples as well as thymocytes were separated by centrifugation and washed twice in PBS to remove unbound monoclonal antibodies. Normal rat plasma was used instead of antibody solution to determine blanks. Fluorescing cells were detected in a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany) at 495 nm excitation and 525 nm emission for FITC-coupled monoclonal antibodies and at 480 and 578 nm for RPE-coupled monoclonal antibodies, respectively. Detection and computing of the data were performed by using the FACScan Research Software (Becton Dickinson). The used monoclonal antibodies are described in *Table 1*. All rat-specific monoclonal antibodies were from Serotec (Oxford, UK). Immunoglobulins G, A, and M in plasma were quantified by radial immunodiffusion according to the instructions of the producer (Rat-RID-Testkits for IgG, IgA, and IgM, Serotec, Oxford, UK). Due to occasional blood clotting, which made determinations of samples impossible, different numbers of rats in various groups resulted.

Statistically significant differences between controls at 3 respectively 6 weeks of age and rats of the Mg- or Zn-deficient groups were determined by single factor analysis of variance (ANOVA, Student's *t*-test) with the aid of the SPSS-software.

Results

Feeding the deficient diets reduced the number of litters and the litter size. In the control group 6 out of 9 rats completed their pregnancy with a mean litter size of 11. In the Mg-deficient group 4 of 9 and in the Zn-deficient group 5 of 14 rats had litters with a mean size of 9 in the Mg-deficient and 8 in the Zn-deficient group. No gross malformations were observed in the Mg-deficient group. Zn deficiency, however, induced severe malformations; six rats were blind without visible eye anlage, in one rat one eye was missing,

Table 1 Used monoclonal antibodies and their specifity

Monoclonal antibody clone	Specifity	Used cells	Marker
MRC OX-19	thymocytes and mature T-cells, no B- or NK-cells	blood, thymus	FITC
MRC OX-7	Thy 1.1 receptor, thymocytes, stem cells and immature B-cells	thymus	RPE
W 3/25	T-helper cells, CD4	blood, thymus	FITC
MRC OX-8	T-suppressor and cytotoxic T-cells, CD8	blood, thymus	RPE
MRC OX-12	B-cells (kappa light chain)	blood	RPE
MRC OX-39	interleukin-2 receptor, activated T-cells, no resting cells	blood	FITC

in other rats 6 weeks after birth no reaction to noise could be observed, indicating deafness, additionally one rat with a curled tail was found. The distribution of males and females was equal in all groups. Body weights of the rats after 3 and 6 weeks are given in *Figure 1*. Rats, subjected to gestational Mg deficiency had a significantly reduced body weight at 3 weeks (−35%) and 6 weeks (−14%) of age, whereas gestational Zn deficiency did not influence postnatal increase of body weight. No postnatal mortality was observed.

Blood

The total number of leukocytes (*Figure 2*) was reduced in Mg deficiency by 25% 3 weeks after birth and by 14% at 6 weeks after birth. This was accompanied by a reduced number of lymphocytes (3 weeks −25%, 6 weeks −42%, *Figure 2*), which represented 75% of total leukocytes in the control but only 64% in Mg-deficient rats. In controls, the number of leukocytes increased by 69% from 3 to 6 weeks and the number of lymphocytes by 80%, whereas in Mg-deficient rats the increase amounted to 94% and 129%. In Zn deficiency, the number and percent increase of leukocytes or lymphocytes was not significantly different from controls.

By FACS analysis it could be shown with the OX 19 monoclonal antibody that 43% (after 3 and 6 weeks) of lymphocytes in controls were mature T-cells, showing the CD5 antigen (*Figure 3*). Of total lymphocytes CD4 cells represented 32% at 3 weeks and 40% at 6 weeks, as detected with the monoclonal antibody W3/15 (*Figure 3*). CD8 (OX 8) positive were 18% of lymphocytes at 3 weeks and 17% at 6 weeks (*Figure 3*). IL-2 receptor (OX 39) bearing cells amounted to 3% at 3 and 6 weeks (*Figure 4*). The relative amounts of B-cells (OX 12) at 3 and 6 weeks were 4% (*Figure 4*).

Significant alterations were not found in the Zn-deficient group with regard to T-cell subsets (*Figures 2–4*), however, the number of B-cells (*Figure 4*) was significantly reduced by 40% 3 weeks after birth, but 6 weeks after birth this alteration was fully compensated.

Significantly lower numbers of CD5, CD4, or CD8 bearing T-cell subsets were found in Mg-deficient rats at 3 weeks (*Figure 3*). After 6 weeks, only CD8 cells were still significantly reduced compared with controls. IL-2 receptor

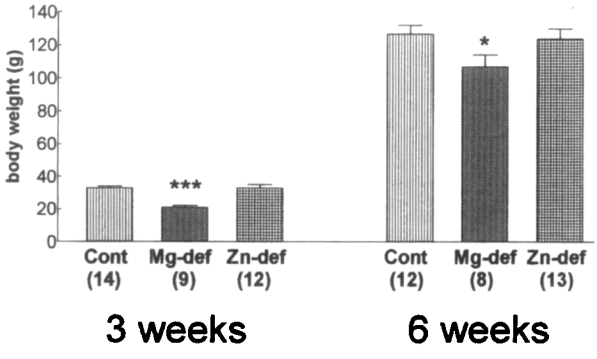

Figure 1 Body weight of rats 3 and 6 weeks after birth. Cont: control; Mg-def: rats subjected to gestational Mg deficiency; Zn-def: rats subjected to gestational Zn deficiency. Mean ± SEM, number of rats in parentheses. Significant differences to controls by ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

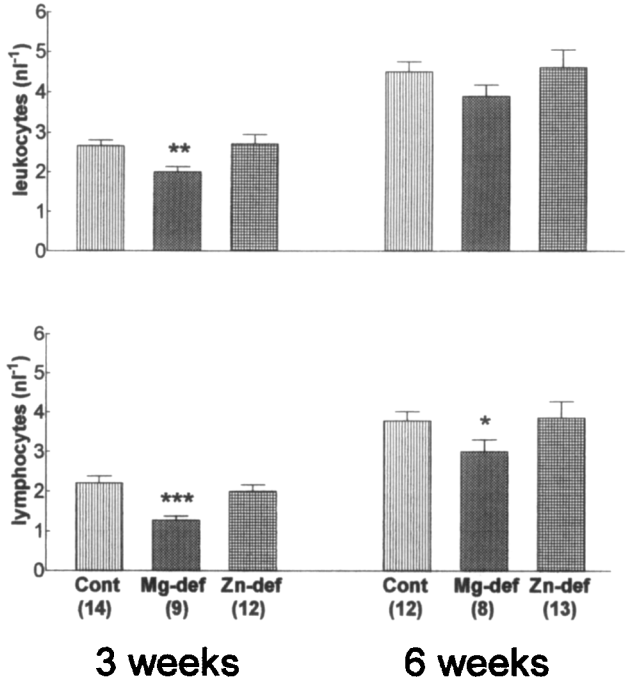

Figure 2 Number of leukocytes and lymphocytes in blood of rats, according to *Figure 1*.

bearing cells and B-cells were not significantly changed (*Figure 4*).

The concentration of IgA in plasma was the same in all groups; there was also no difference between 3 and 6 weeks (*Figure 5*).

In controls, the content of IgG in plasma dropped from 6.5 to 2.2 g/l from week three to week six. After 6 weeks, plasma IgG content in Mg-deficient rats was 17% and in Zn-deficient rats 24% lower than in controls (*Figure 5*).

IgM in control plasma rose from 0.35 to 0.45 g/l between weeks three and six. Zn-deficient rats had a 36% reduced IgM content 3 weeks after birth (*Figure 5*). Mg deficiency did not significantly influence IgM in plasma.

Thymus

The percentage of cells bearing the respective antigens is shown in *Table 2*. More than 90% of all isolated thymocytes expressed CD4, CD5, CD8, or Thy 1.1 on their surface. No significant differences among the groups could be observed.

Discussion

Zn deficiency

Gestational Zn deficiency reduced litter number and size as has previously been reported.[18] The degree of Zn deficiency was high enough to induce severe malformations as indicated by the literature.[6] An additional unreported finding was the induction of deafness.[a] These fetotoxic events indicate severe Zn deficiency during gestation; in spite of this

[a]further details will be published

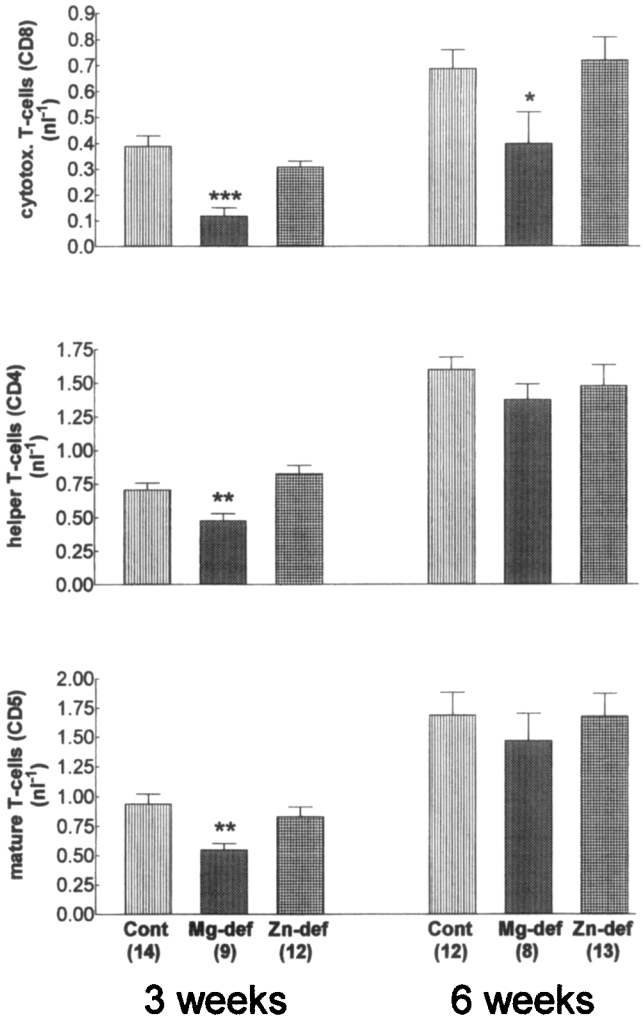

Figure 3 Number of mature T-cells (CD5), helper T-cells (CD4), and cytotoxic T-cells (CD8) in blood of rats, according to *Figure 1.*

offspring from Zn-deficient dams developed normally after birth. Reduced gestational Zn supply did not influence the total number of leukocytes and lymphocytes in blood either with the exception of the small number of B-cells. A change in T-cell subsets could not be detected. The number of B-cells was reduced 3 weeks after birth; this correlates with the reduced concentration of IgM in plasma. After 6 weeks, the number of B-cells was no longer significantly different from controls and also IgM was normalized, indicating a repair of B-cell function, which after maturation to plasma cells at first produce IgM. The switch from IgM to IgG production, however, seems to be delayed because 6 weeks after birth, IgG concentration in plasma was significantly reduced compared with controls. Another reason for this effect might be an increased degradation of maternal IgG, which physiologically occurs between weeks three and six as observed in the controls.

These data in rats are in contrast to results obtained with mice,[9] where a drastic reduction of IgM, IgG_{2a} and IgA production was observed. Reduced amounts of IgM persisted for three generations after gestational Zn deficiency. Also in rhesus monkeys gestational Zn deficiency reduced humoral immunity in the newborns.[21] A changed methylation pattern of immunoregulatory genes during Zn deficiency was discussed to be a possible reason for this long lasting effect.[22] It is quite possible that this is a species-specific effect that is not present in rats even though other symptoms (such as malformations) clearly showed the existence of severe Zn deficiency. In rats there may be a higher rate of repair mechanisms, leading to normalization of the reduced IgM content as early as 6 weeks after birth. Additionally, in mice due to a higher percentage of fetal compared to maternal body mass, Zn deficiency might be more expressed.

Mg deficiency

Mg deficiency significantly reduced the weight of the 3- and 6-week-old rats, although at day 21 of gestation only a small effect of Mg deficiency on fetus weight could be detected.[18] In correlation to body weight, also total numbers of leukocytes and lymphocytes were reduced. The reduction of lymphocytes was mainly caused by a diminished content of T-cells; CD8-cells were reduced to the highest extent. Six weeks after birth, the differences between Mg-deficient rats and controls became less significant or were no longer detectable. Thereafter, the reduced number of lymphocytes was mainly caused by a significantly reduced number of CD8-cells. It is reasonable to assume that the low number of T-cells is caused by the reduction in absolute and relative thymic weights after gestational Mg deficiency as has been reported by Kubena et al.[17] In their experiments 17 days after birth the immune response in pups, which were subjected to gestational Mg deficiency, was not normalized after challenge with sheep red blood cells.

The populations of thymocytes do not indicate any sig-

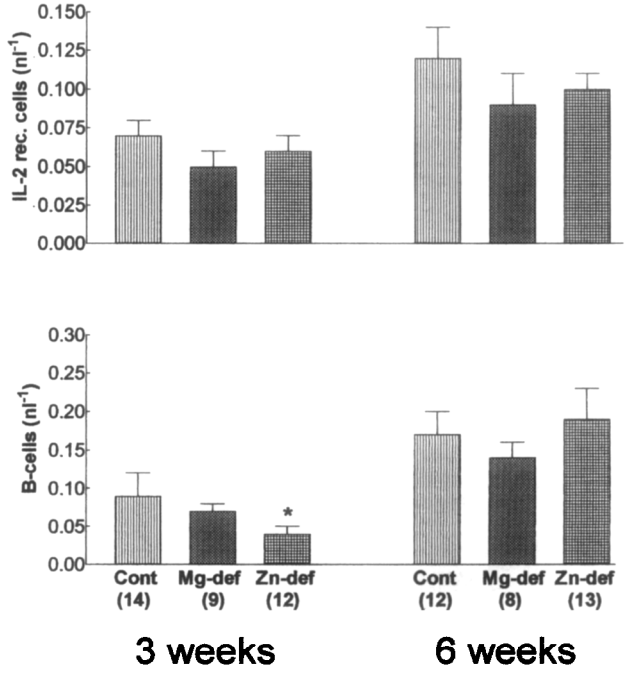

Figure 4 Number of interleukin-2 receptor-bearing cells and B-cells in blood of rats, according to *Figure 1.*

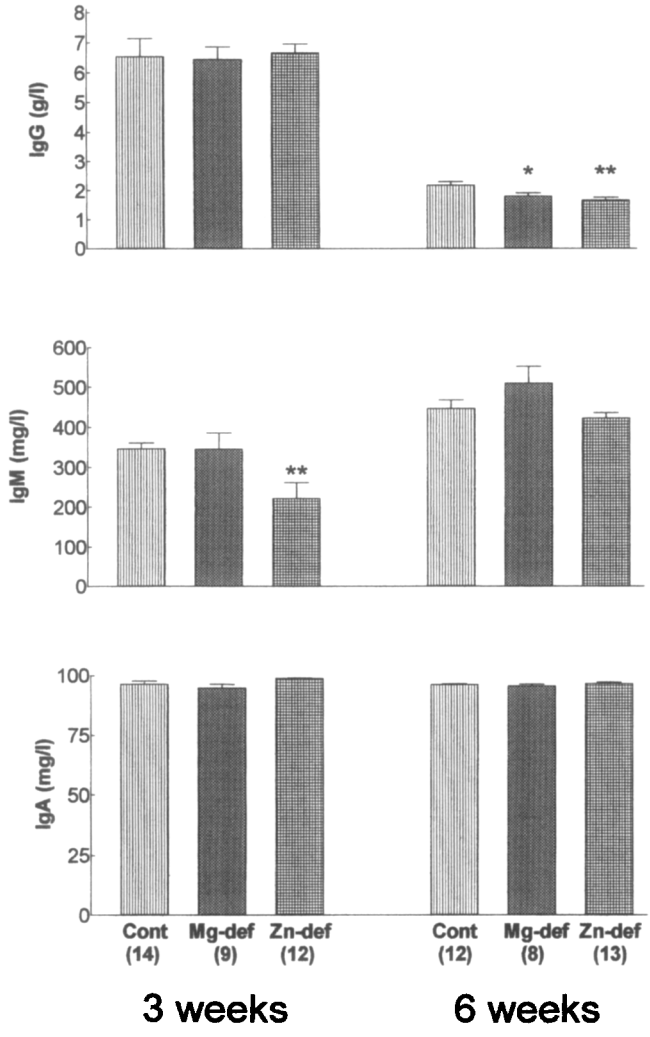

Figure 5 IgA, IgM and IgG in plasma of rats, according to *Figure 1*.

nificant shift in the thymus-dependent maturation of T-cells in Mg and Zn deficiency.

The pronounced reduction in the number of peripheral cytotoxic T-cells in our experiment may suggest that in addition to reduced humoral immunity, the immunity towards virus-infected or transformed cells also may be impaired.

Table 2 Percentage of marker bearing isolated rat thymocytes in 3- and 6-week-old rats (same animal numbers as in *Figure 1*). Mean ± SEM

Monoclonal antibody clone	Control	Mg-def.	Zn-def.
OX 19 (CD5)			
3 weeks	96.3 ± 1.3	94.9 ± 1.4	98.8 ± 0.2
6 weeks	98.1 ± 0.4	98.0 ± 0.4	99.0 ± 0.3
W3/25 (CD4)			
3 weeks	95.1 ± 1.4	92.9 ± 1.6	96.1 ± 0.2
6 weeks	96.2 ± 0.2	95.7 ± 0.5	96.6 ± 0.5
OX 8 (CD8)			
3 weeks	93.2 ± 0.8	91.4 ± 1.2	93.6 ± 0.5
6 weeks	91.3 ± 0.7	91.3 ± 0.5	91.4 ± 0.8
OX 7			
3 weeks	97.3 ± 0.9	94.8 ± 1.8	98.9 ± 0.9
6 weeks	98.8 ± 0.2	98.2 ± 0.4	96.4 ± 1.9

Similarly to gestational Zn deficiency, IgG content was reduced in 6-week-old rats also after gestational Mg deficiency. As significant changes in other immunoglobulins or B-cells could not be detected, this might be caused by a reduced lifetime of maternal IgG.

The reduced immunity after gestational Mg deficiency, however, may be an unspecific effect caused by the overall intrauterine growth retardation. Reduced birth weight due to various reasons is known to depress cell-mediated immunity (as reviewed in [4]).

In conclusion, gestational Mg or Zn deficiency led to expressed effects on the fetuses such as reduced litter number and litter size in both deficient states, body weight reduction in Mg deficiency and induction of malformations in Zn deficiency. Effects on immunity primarily concern peripheral T-cells in Mg deficiency, whereas in Zn deficiency B-cell number in blood is reduced and B-cell function may be impaired. Under normal animal housing conditions with no additional immune challenge no effect of gestational Mg or Zn deficiency on the number of activated T-cells (bearing IL-2 receptors) was found.

With the exception of plasma IgG content, effects of gestational Mg or Zn deficiency on immunity either improved significantly from week three to six or were already normalized within 6 weeks after birth.

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