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Inflammatory response following acute magnesium deficiency in the rat

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Abstract

The importance of inflammatory processes in the pathology of Mg deficiency has been recently reconsidered but the sequence of events leading to the inflammatory response remains unclear. Thus, the purpose of the present study was to characterize more precisely the acute phase response following Mg deficiency in the rat. Weaning male Wistar rats were pair-fed either a Mg-deficient or a control diet for either 4 or 8 days. The characteristic allergy-like crisis of Mg-deficient rats was accompanied by a blood leukocyte response and changes in leukocytes subpopulations. A significant increase in interleukin-6 (IL-6) plasma level was observed in Mg-deficient rats compared to rats fed a control diet. The inflammatory process was accompanied by an increase in plasma levels of acute phase proteins. The concentrations of α2-macroglobulin and α1-acid glycoprotein in the plasma of Mg-deficient rats were higher than in control rats. This was accompanied in the liver by an increase in the level of mRNA coding for these proteins. Moreover, Mg-deficient rats showed a significant increase in plasma fibrinogen and a significant decrease in albumin concentrations. Macrophages found in greater number in the peritoneal cavity of Mg-deficient rats were activated endogenously and appeared to be primed for superoxide production following phorbol myristate acetate stimulation. A high plasma level of IL-6 could be detected as early as day 4 for the Mg-deficient diet. Substance P does not appear to be the initiator of inflammation since IL-6 increase was observed without plasma elevation of this neuropeptide. The fact that the inflammatory response was an early consequence of Mg deficiency suggests that reduced extracellular Mg might be responsible for the activated state of immune cells.

Keywords: Magnesium; Inflammation; Free radical; Acute phase protein; Rat

1. Introduction

Magnesium plays an essential role in fundamental cellular reactions [1] and the importance of the immunoinflammatory processes in the pathology of Mg deficiency has recently been reconsidered [2]. In 1932, Kruse et al. [3] reported peripheral vasodilatation

Abbreviations: TNF, tumor necrosis factor; IL, interleukin; PMA, phorbol myristate acetate; α2-M, α2-macroglobulin; α1-AGP, α1-acid glycoprotein

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with hyperemia as part of the symptoms caused by Mg deficiency in rats. Thereafter, many investigators have recognized the same symptoms in relation to degranulation of mast cells with a release of histamine and inflammatory mediators [4,5]. The response is analogous to an anaphylactoid reaction. The possibility that an inflammatory response induced by Mg deficiency was initiated by an early neurogenic inflammatory process through the secretion of substance P was recently suggested [6]. This inflammatory response has been proposed to be responsible for oxidative damage in Mg deficiency [7] but the sequence of events leading to the inflammatory response remains unclear. Thus, the aim of the present study was to characterize more precisely this inflammatory response.

2. Materials and methods

2.1. Experimental animals

Male weaning Wistar rats (Iffa-Credo, L'Arbresle, France) weighing 61 ± 3 g were randomly divided into Mg-deficient and control groups. The institution's guide for the care and use of laboratory animals was used. The rats were housed in wire-bottomed cages in a temperature-controlled room (22°C) with a 12 h dark (20.00–08.00 h) and 12 h light period. They were pair-fed with the appropriate diets using an automatic feeding apparatus and maintained on the experimental diet for either 4 or 8 days. Distilled water was provided ad libitum. The synthetic diets contained (g/kg): 200 casein, 650 sucrose, 50 corn oil, 50 alphacel, 3 DL-methionine, 2 choline bitartrate, 35 modified AIN-76 mineral mix and 10 AIN-76A vitamin mix (ICN Biomedicals, Orsay, France). MgO was omitted from the mineral mix in the Mg-deficient diet. The Mg concentrations of the diets, determined by flame atomic absorption spectrometric analysis (Perkin Elmer 400, Norwalk, CT, USA), were 32 and 950 mg/kg respectively, for deficient and control diets. Experiments were performed in rats fed experimental diets for 8 days (12 control and 12 deficient rats). Additional groups of 12 control and 12 deficient rats were killed for Mg, cytokine and substance P measurements in the plasma after 4 days on the experimental diets. Non-fasted rats were weighed, anesthetized with sodium pentobarbital (40 mg/kg of body weight, i.p.) and killed. Blood was collected from the abdominal aorta into heparinized tubes. Plasma obtained after low-speed centrifugation (2000 × *g* for 15 min) was stored at −80°C for biochemical analyses. The liver was removed after blood sampling.

2.2. Hematological analysis

The number of total white cells was determined by a cell counter (Cobas, Hoffmann La Roche). The differential leukocyte counts for polymorphonuclear cells, monocytes and lymphocytes were made from a blood smear stained with the May–Grünwald and Giemsa stain (Sigma, St. Quentin Fallavier, France). The separation of blood mononuclear cells on a gradient density, the immunolabelling of mononuclear cell surface markers and subsequent analysis by flow cytometry (FCM) were performed as previously described [8].

2.3. Minerals

Plasma magnesium and zinc were determined by flame atomic absorption spectrometric analysis (Perkin Elmer 400).

2.4. Cytokines and substance P measurements

Tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) concentrations were measured using bioassays with L929a, a fibroblast cell line, and B9, a murine hybridoma cell line [9], respectively. The standards used were serially diluted human recombinant TNFα or IL-6 (NIBSC, Hertfordshire, UK). The circulating level of substance P (SP) was measured by a radioimmunoassay after 4 and 8 days on the experimental diet, according to a defined protocol (Peninsula Laboratories) using rabbit antiserum. The cross-reactivity with related peptides was less than 0.01%. The sensitivity of the SP assay was 1 fmol/tube. Briefly, the radioimmunoassay was performed in a phosphate buffer (19 mM monobasic and 81 mM dibasic sodium phosphate, pH 7.4; 0.05 M NaCl; 0.1% bovine serum albumin; 0.1% Triton X-100 and 0.01% NaN_3). 100 μl of standard or plasma was incubated overnight at 4°C with

100 μl of antiserum, ^{125}I-Tyr^{8}-SP (15 000 cpm/100 μl) was added and it was again incubated overnight at 4°C. The reaction was stopped by adding 100 μl of goat anti-rabbit IgG serum and 100 μl of normal rabbit serum. Precipitates were allowed to form for 2 h at room temperature, were then centrifuged at 1700×*g* for 20 min and the pellets were counted for ^{125}I.

2.5. Acute phase proteins

α2-Macroglobulin (α2-M) and α1-glycoprotein acid (α1-AGP) plasma levels were measured using rocket immunoelectrophoresis as previously described [10]. The standards utilized for establishing a standard curve were, respectively, the α2-M level found in rats injected with turpentine 48 h before (100% reference) and purified α1-AGP. The hepatic mRNA levels of these proteins were analyzed as described by Lyoumi et al. [10].

The fibrinogen concentration in the plasma was measured using a commercial kit (Fibri-Prest, bio-Mérieux, Marcy-l'Etoile, France) based on the principle that the time taken for coagulation of the plasma reflects the concentration of fibrinogen. Plasma albumin concentrations were measured by radial immunodiffusion [11] using a rabbit anti-rat albumin antibody made in our laboratory.

2.6. Activity of peritoneal cells

Peritoneal resident cells were obtained aseptically by peritoneal washes with 15 ml of cold Hanks' balanced salt solution without phenol red (modified HBSS) (Sigma). Macrophages were obtained by seeding the peritoneal suspension for 3 h at 37°C. Cells were then gently removed and washed with modified HBSS (Sigma) by centrifuging the cell suspension at 500×*g* for 15 min. Finally, the cells were suspended in modified HBSS at 10^{7} cells/ml and chemiluminescence was performed to measure the respiratory burst. Briefly, 100 μl of luminol (Sigma) at 10^{-4} M was added to 100 μl of macrophage suspension with or without 100 μl of phorbol myristate acetate (PMA) (Sigma) at 10 μg/ml. The chemiluminescence was measured at 37°C using a luminometer LKB Wallac 1251 (Wallac OY, Turku, Finland).

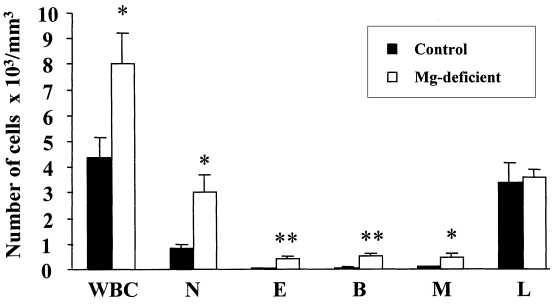

Fig. 1. Number of leukocytes in blood of control and Mg-deficient rats after 8 days on the experimental diets. Means ± S.E.M. of 12 rats per group. *$P<0.05$; **$P<0.01$. WBC: total white blood cells; N: polymorphonuclear neutrophils; E: polymorphonuclear eosinophils; B: polymorphonuclear basophils; M: monocytes; L: lymphocytes.

2.7. Statistical methods

Results are expressed as means ± S.E.M. The statistical significance of differences between groups was assessed using Student's *t* unpaired test (Instat, Graph pad, San Diego, CA, USA). Results were considered significant at $P<0.05$.

3. Results

After 8 days on the experimental diet, Mg-deficient rats display the usual decrease in plasma Mg (0.14 ± 0.02 versus 0.81 ± 0.01 mM, $P<0.01$) and suffered a small weight reduction compared with the control animals (90 ± 3 versus 96 ± 3 g, $P>0.05$). Peripheral vasodilatation with hyperemia

Table 1
Expression of the surface antigens of lymphocytes in blood of control and Mg-deficient rats after 8 days on the experimental diets

	Dietary group (% positive cells)	
	Control	Mg-deficient
LCA	83.8 ± 5.3	75.6 ± 8.9
Thy 1	80.1 ± 1.8	75.4 ± 4.8
CD5	70.4 ± 3.6	56.5 ± 6.4
CD4	52.4 ± 5.5	42.8 ± 5.9
CD8	20.7 ± 2.4	15.2 ± 2.5
Pan B	11.2 ± 2.3	12.4 ± 2.0

Values are means ± S.E.M., $n=8$. LCA: leukocyte common antigen; Thy 1: isoantigen present on the surface of thymus-derived lymphocytes; CD: cluster of differentiation; Pan B: surface epitopes found on all B lymphocytes.

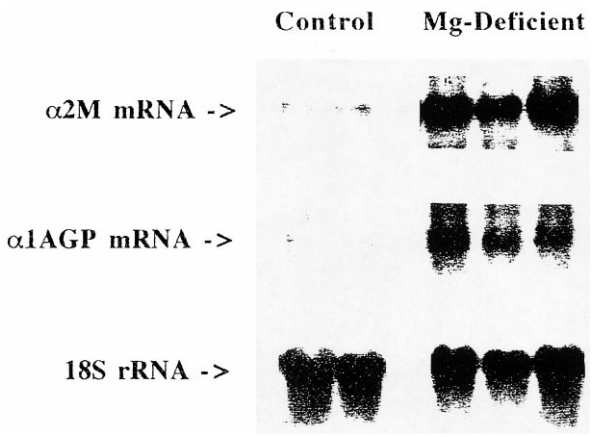

Fig. 2. Hepatic α2-M and α1-AGP abundance in control and Mg-deficient rats after 8 days on the experimental diets. Northern blot analysis; 20 μg of total mRNA was fractionated through 1% agarose-formaldehyde gels prior to transfer to a nylon membrane. The membrane was probed for α2-M, α1-AGP and β-actin mRNAs.

of the ears was apparent in all Mg-deficient rats. In the blood, the inflammatory response was associated with a marked increase in the total circulating leukocytes predominantly in the neutrophil fraction. Monocytes, eosinophils and basophils also increased (Fig. 1). Total circulating lymphocyte levels were not modified, but a non-significant decrease in the proportion of CD5+ and CD8+ lymphocytes was observed (Table 1). Higher plasma IL-6 levels were found in Mg-deficient rats compared to control rats but the TNFα plasma levels remained below the limit of detection in both groups. The concentrations of α2-M and α1-AGP in the plasma were higher than that of control rats (Table 2). This was accompanied in the liver by an increase in the level of mRNA coding for these proteins (Fig. 2). Moreover, Mg-deficient rats showed a significant increase in plasma fibrinogen levels and a decrease in the plasma level of albumin and zinc (Table 2).

When peritoneal cells were isolated by peritoneal washes with saline, the number of cells obtained from Mg-deficient rats was about two-fold greater than in controls. In both groups, the proportion of macrophages was about 60% of the total cell population and the mean number of macrophages recovered in Mg-deficient animals was significantly higher than in controls rats (Table 3). Histological examination indicates the presence of activated macrophages as shown by an increased cell volume and an increased number of vacuoles (data not shown). To measure the respiratory burst of macrophages, chemiluminescence was monitored. The chemiluminescent activity of resident macrophages from Mg-deficient rats was higher than that of control rats (Fig. 3). After in vitro PMA stimulation, the chemiluminescent activity of macrophages from Mg-deficient rats was four-fold higher as compared to cells from control rats, and superoxide generation was markedly increased in the same way (Fig. 3). Together, these results indicate that in Mg-deficient rats, the phagocytic cells are metabolically stimulated.

Further observations were made in rats following

Table 2
Inflammatory cytokines, acute phase proteins and zinc concentrations in plasma of control and Mg-deficient rats after 8 days on the experimental diets

	Dietary group	
	Control	Mg-deficient
IL-6 (pg/ml)	39 ± 2	98 ± 12***
TNFα (pg/ml)	n.d.	n.d.
α2-M (a.u.)	2.10 ± 0.03	19.1 ± 1.8***
α1-AGP (mg/ml)	0.07 ± 0.01	0.13 ± 0.01***
Fibrinogen (g/l)	1.90 ± 0.06	2.54 ± 0.15**
Zn (mg/100 ml)	114.7 ± 6.8	85.4 ± 3.8**
Albumin (mg/ml)	38.1 ± 3.3	27.3 ± 1.2**

n.d.: not detectable; a.u.: arbitrary units. Values are means ± S.E.M., $n = 12$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

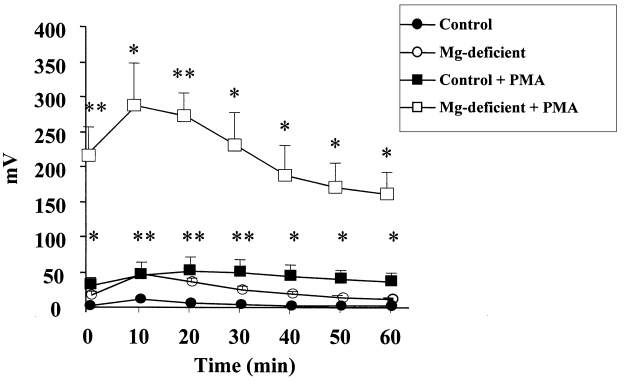

Fig. 3. Macrophage activation in control and Mg-deficient rats after 8 days on the experimental diets. The macrophages were isolated as described in Section 2. Their reactive oxygen producing activities were determined by chemiluminescence, basal or in the presence of PMA. Means ± S.E.M. of eight rats per group. *$P < 0.05$; **$P < 0.01$.

Table 3
Number and percent distribution of cells obtained after washing the peritoneal cavity of control and Mg-deficient rats with physiological saline after 8 days on the experimental diets

	Dietary group	
	Control	Mg-deficient
Peritoneal cell count $\times 10^6$	5.8 ± 0.5	13.8 ± 1.2*
Distribution (%)		
Macrophages	58.0 ± 1.3	61.0 ± 3.9
Neutrophils	1.4 ± 0.3	1.4 ± 0.5
Eosinophils	3.6 ± 1.8	2.6 ± 1.2
Mastocytes	3.4 ± 0.6	4.4 ± 1.2
Lymphocytes	33.6 ± 2.9	30.6 ± 5.1

Values are means ± S.E.M., $n = 12$. *$P < 0.05$.

4 days of Mg deprivation. The body weights of deficient and control rats were similar. Hypomagnesemia was observed (0.15 ± 0.01 versus 0.83 ± 0.02 mM; $P < 0.01$). Mg deficiency did not result in hyperemia and leukocytosis but Mg-deficient rats had higher plasma IL-6 values than control animals. There were no significant differences in the plasma TNFα and SP values between control and deficient animals (Table 4).

4. Discussion

This study confirms the occurrence of an inflammatory response in Mg-deficient rats but this experiment is, to our knowledge, the first demonstration that Mg deficiency in a rodent model results after a few days in the activation of macrophages and an elevation of the plasma concentration of IL-6, a known mediator of the acute phase response. A characteristic allergy-like crisis occurs spontaneously in Mg-deficient rats, the first visible symptom being a peripheral vasodilatation of the ears. This hyperemia crisis was observed by Kruse et al. [3] and has been considered to be related to the elevation of blood histamine levels, which derives from the degranulation of mast cells [12]. A blood leukocyte response and changes in leukocyte subpopulations are also consequences of Mg deficiency [13,14]. Neutrophils are a well-known prominent cell type during acute inflammation and an elevated circulating level of eosinophils is a usual response to mast cell degranulation and histamine secretion. These marked alterations in circulating inflammatory cells may arise due to cellular hyperplasia of cell lines in the bone marrow [15]. These changes are not accompanied by a rise in lymphocyte counts and the slight decrease in the proportion of CD5 and CD8 might reflect a decrease of humoral immunity concomitant with thymus alterations [16].

Table 4
Cytokines and SP concentrations in plasma of control and Mg-deficient rats, after 4 days on the experimental diets

	Dietary group (day 4)	
	Control	Mg-deficient
IL-6 (pg/ml)	45 ± 2	119 ± 30*
TNFα (pg/ml)	n.d.	n.d.
SP (fmol/ml)	45.7 ± 6.1	42.5 ± 2.2

n.d., not detectable: values below 2.5 pg/ml as assayed by bioassay. Results are mean ± S.E.M., $n = 12$. *$P < 0.05$.

Elevated levels of proinflammatory cytokines (IL-6, TNFα) have been observed by Weglicki et al. [17] in Mg-deficient animals following 3 weeks of Mg deprivation. In this experimental model, plasma SP was elevated during the first week of Mg deficiency. This 'neuronal' tachykinin is thought to be released from neural tissues and it is known to stimulate the production of cytokines. Based on the fact that SP increased before cytokine elevation, it has been hypothesized that the release of SP may be the earliest pathophysiological event leading to stimulation of the inflammatory cytokine in Mg deficiency [6]. Our own studies confirm that Mg deficiency elevates circulating levels of IL-6 but there was a non-concomitant increase in TNFα. Moreover, the time course of the inflammatory response is quite different from that in the previous study [17]. In the present study, high levels of IL-6 could be detected as early as day 4 in animals on a Mg-deficient diet without a significant increase in the circulating level of SP. It may be concluded that the increase in IL-6 is primarily induced by Mg depletion and is not due to an increase in the plasma SP concentrations. We were unable to detect TNFα in the plasma of control and Mg-deficient rats. Using the same experimental model, we have shown that Mg-deficient rats had significantly higher TNFα plasma values than control rats following endotoxin challenge [18]. Thus, this specific

cytokine may be elevated following immune stress. TNFα is generated in sufficient amounts to reach detectable levels in the blood of control and Mg-deficient rats. However, higher TNFα levels were found in Mg-deficient rats compared to control rats following endotoxin challenge in the same experimental model [18]. Although IL-6 acts on many different cell types, the liver seems to be one of the major target organs for IL-6 in vivo and IL-6 induces acute phase protein synthesis during the inflammatory response [19]. Thus, Mg deficiency is associated with an increase of α2-M, α1-AGP and fibrinogen in the plasma. In contrast the albumin concentration decreases in the plasma of Mg-deficient rats. The inflammatory response is also associated with alterations in trace elements. IL-6 may stimulate hepatic metallothionein synthesis leading to sequestration of Zn by the liver and resulting in a decrease in plasma Zn concentration [20]. The inflammatory response in Mg-deficient rats suggests that Mg deficiency might be accompanied by the activation of a number of cells including macrophages, neutrophils and endothelial cells [21,22]. Accordingly, Mg deficiency results in neutrophil and endothelial cell activation. In the present study, macrophages were found in greater numbers in the peritoneal cavity of Mg-deficient rats. Histological examination and chemiluminescent activity suggest that macrophages from Mg-deficient rats are activated endogenously. Moreover, superoxide anion production following PMA stimulation was higher in macrophages from Mg-deficient as compared to control animals, i.e. these cells had an enhanced response to stimulation. These results are consistent with the notion that increased oxidative stress occurs during the inflammatory response in Mg-deficient animals [7] and that macrophages, at least in part, contribute to the increased production of inflammatory cytokines. The proinflammatory cytokines and oxidant molecules produced during the inflammatory response may be beneficial or detrimental to the host depending on the amounts and contexts in which they are produced. Increased thiobarbituric acid reactive substance levels in the plasma and NO overproduction have been shown in Mg-deficient rats [23,24]. Tissues of Mg-deficient rats have a greater tendency to undergo lipid peroxidation than do tissues of control animals [7]. Increased production of free radicals can result in loss in membrane integrity and oxidative injury in Mg deficiency [7]. Young animals fed diets deficient in Mg frequently develop cardiomyopathic lesions [6]. Data from Weglicki et al. [25] support a free radical participation in cardiomyopathy due to Mg deficiency and an inflammatory sequence that results in cardiac tissue necrosis. Mg deficiency also leads to skeletal muscle damage that could also be the consequence of free radical injury [26]. Other studies suggest that the inflammatory response in Mg deficiency might contribute to atherosclerotic processes by modifications of lipoprotein metabolism, inflammatory cell recruitment, oxidative modification of lipoproteins and by the release of several growth factors that induce cell migration and proliferation [22,27–29]. Moreover, the enhanced response of macrophages to stimulation, as shown in the present study, might explain the vulnerability of Mg-deficient rats to endotoxin challenge [18].

The underlying mechanism for the activation of inflammatory cells in Mg-deficient animals is unknown. However, the present study indicates that the activated state of immune cells is an early event occurring after a few days of Mg deficiency. Since the cellular Mg content is tightly regulated and changes only slightly even when the extracellular concentration is drastically decreased [30], total intracellular Mg is probably unaffected in experimental conditions. Thus, the effect of Mg deficiency may be induced by the reduction of the extracellular Mg^{2+} concentration. The pathophysiological response to the immune stress includes activation of several processes, which are dependent on cytosolic Ca^{2+} elevation [31]. Since Mg frequently acts as a natural Ca antagonist [32], additional studies are needed to determine if the proinflammatory effect of Mg deficiency is the consequence of a reduced extracellular Mg^{2+}/Ca^{2+} antagonism resulting in an increased intracellular free Ca^{2+} concentration.

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