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Increased phagocytosis and production of reactive oxygen species by neutrophils during magnesium deficiency in rats and inhibition by high magnesium concentration

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Recent studies underline the importance of the immunoinflammatory processes in the pathology of Mg deficiency. Neutrophils possess a superoxide anion-generating NADPH oxidase and its inappropriate activation may result in tissue damage. The aim of the present study was to assess the effect of experimental Mg deficiency in the rat on polymorphonuclear leucocytes (PMN) activity and the role of increasing extracellular Mg. Weaning male Wistar rats were fed either a Mg-deficient or a control diet for 8 d. In Mg-deficient rats, the characteristic inflammatory response was accompanied by a marked increase in the number of PMN. Higher plasma interleukin 6 and NO concentrations and increased lipid peroxidation in the heart were found in Mg-deficient rats as compared with control rats. As shown by chemiluminescence studies, basal neutrophil activity from Mg-deficient rats was significantly elevated when compared with neutrophils from control rats. Moreover, the chemiluminescence of PMN from Mg-deficient rats was significantly higher than that of control rats following phorbol myristate acetate or opsonized zymosan activation. PMN from Mg-deficient rats also showed an increased activity of phagocytosis in comparison with neutrophils from control animals. Increasing extracellular Mg concentration in the incubating medium of PMN (0·8 *v.* 8·0 mM) decreased the chemiluminescence activity of PMN from control rats following opsonized zymosan activation. Chemiluminescence activities of PMN from Mg-deficient rats following phorbol myristate acetate or opsonized zymosan challenge were also decreased by high extracellular Mg concentration. From this work, it appears that PMN activation is an early consequence of Mg deficiency and that high extracellular Mg concentration inhibits free radicals generation.

Magnesium: Inflammation: Polymorphonuclear leucocytes

Mg, the second most abundant intracellular cation, plays an essential role in a wide range of fundamental cellular reactions and many signs, symptoms and disease states are attributed to altered Mg homeostasis (Shils, 1994). In developed countries, the marginal Mg intake may induce a high prevalence of marginal Mg deficiency (Galan *et al.* 1997). Mg deficiency has been extensively studied in the rat, where it is readily produced by dietary depletion. In this experimental model, several studies have provided evidence that Mg deficiency causes abnormal tissue sensitivity to oxidative stress (Rayssiguier *et al.* 1993). A particular type of cardiomyopathy has been shown in Mg-deficient animals and it has been considered as the result of free radicals damage (Freedman *et al.* 1990). A free radical pathogenesis has also been hypothesized in several human cardiovascular diseases associated with Mg-deficient status (Rayssiguier *et al.*1997).

Hyperaemia of the ears is the first visible symptom of the experimental Mg-deficiency in rats (Kruse *et al.* 1932). The metabolism of mast cells and histamine has received much attention since several authors related the characteristic inflammation of Mg-deficiency to increased histamine

Abbreviations: HBSS, Hank's balanced salt solution; PMA, phorbol myristate acetate; PMN, polymorphonuclear leucocyte.

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production (Bois *et al.* 1963). Recent studies from our laboratory as well as from others suggest that in Mg deficiency, phagocytes are metabolically stimulated and produce reactive oxygen species leading to tissue damage. The response to Mg-deficiency is accompanied by increased production of NO and inflammatory cytokines (Weglicki & Phillips, 1992; Malpuech-Brugère *et al.* 2000). However, the precise mechanism of this characteristic response to Mg-deficiency remains to be clarified. Neutrophils possess a superoxide anion generating NADPH oxidase and its inappropriate activation may result in tissue damage in inflammatory conditions (Babior, 1984). Moreover, the possibility exists that extracellular Mg may influence neutrophil function (Simchovitz *et al.* 1990). Thus, the purpose of the present study was to assess the phagocytic activity and superoxide anion production of blood neutrophils in Mg-deficient rats compared with control animals and the effect of *in vitro* exposure to Mg.

Materials and methods

Experimental design

Male weaning Wistar rats weighing 61 (SE 5) g were randomly divided into Mg-deficient and control groups containing fifty-three and fifty rats respectively. They were maintained from a colony of laboratory animals of the National Institute of Agronomic Research (INRA, Clermont-Ferrand/Theix, France). 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 light–dark cycle (lights on at 08.00 hours). They were maintained on the experimental diets for 8 d. Distilled water and diet were provided *ad libitum*. The synthetic diets contained (g/kg): casein 200, sucrose 650, corn oil 50, alphacel 50, DL-methionine 3, choline bitartrate 2, modified AIN-76 mineral mix 35, AIN-76A vitamin mix 10 (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, were 20 and 950 mg/kg for Mg-deficient and control diets respectively. Rats were anaesthetized with pentobarbital (40 mg/kg body weight). Blood was collected from the abdominal artery in heparinized and sterile tubes. Plasma obtained after centrifugation (2000 ***g*** for 15 min) was stored at −80°C for biochemical analysis. Spleen and hearts were removed. Hearts were kept frozen at −80°C before performing the lipid peroxidation assay.

Polymorphonuclear leucocyte isolation

Polymorphonuclear leucocytes (PMN) were isolated using a double-layer discontinuous gradient of Histopaque®-1083/1119 (Sigma Diagnostics, St Louis, MO, USA). After centrifugation (700 ***g***, 30 min, 20°C), the layer of PMN was collected and contaminating erythrocytes were lysed by exposure to hypotonic saline. PMN were washed in Mg–Ca-free buffer and resuspended in modified Hank's balanced salt solution (HBSS; mM: calcium chloride 1·3, magnesium sulfate 0·8, potassium chloride 5·4, potassium phosphate monobasic 0·44, sodium bicarbonate 4·2, sodium chloride 137, sodium phosphate dibasic 0·34, glucose 5·6, pH 7·3; Sigma Diagnostics). The purity of neutrophil suspensions averaged 90 % as assessed by May-Grünwald staining. Viability averaged 99 % as tested by Trypan Blue exclusion and was not affected by incubation conditions.

Chemiluminescence studies

Free radical generation from PMN was measured using a luminometer (LKB-1251; Wallac OY, Turku, Finland). The method for measurement was similar to that described earlier (Wang *et al.* 1993) with slight modifications. In brief, 3×10^5 PMN were placed in HBSS buffer, pH 7·4 and luminol (Sigma Diagnostics) at a final concentration of 0·2 mM was added. The final volume of the assay mixture was 1000 μl. The peak of chemiluminescence was recorded and expressed in mV. Opsonized zymosan (Sigma Diagnostics) was prepared as previously described by Hasegawa *et al.* (1997). An experiment was performed using eight control and eight Mg-deficient rats in order to assess the effect of Mg deficiency on PMN basal activity and on PMN response to phorbol myristate acetate (PMA Sigma Diagnostics; 1·23 μg/ml) and opsonized zymosan (1 mg/ml). Other experiments were performed in order to assess the effect of a high concentration of extracellular Mg (8·0 *v.* 0·8 mM) on PMN response. In a first experiment, chemiluminescence activity of PMN from control rats (*n* 5) was assessed following opsonized zymosan activation. Finally, a second experiment was performed using Mg-deficient animals (*n* 8) in order to assess the effect of high concentration of extracellular Mg on PMN response to PMA and opsonized zymosan.

Phagocytosis assay

Phagocytosis studies were performed using five control and five Mg-deficient rats. Bovine septicaemic *E. coli* strain 31A ($O153:K^-:H^-$) was used for all experiments. After growth in a Minca medium, bacteria were collected by centrifugation (1500 ***g***, 10 min) and washed with HBSS, bacteria cells were opsonized by normal rodent sera (200 ml/l HBSS) for 30 min at 37°C, washed with HBSS and diluted to a concentration of 10^7 cells/ml with HBSS containing appropriate antibiotics.

Neutrophils were pelleted by centrifugation and resuspended in RPMI 1640 (Sigma Diagnostics) supplemented with fetal calf serum (100 ml/l). PMN (4×10^5) were dispensed in wells of Lab-Teck chamber slides (Nunc Inc., Napperville, IL, USA) and allowed to adhere for 30 min in a humidified 5 % CO_2 incubator at 37°C. Chambers were washed with RPMI 1640 (Sigma Diagnostics) to eliminate non-adherent cells. Opsonized bacteria (4×10^6) were incubated with PMN for 90 min in 5 % CO_2 incubator at 37°C. Then, the association and phagocytosis of bacteria were determined after staining with a LIVE/DEAD bacLight bacterial viability kit (Molecular Probes, Leiden, The Netherlands). The neutrophil-associated bacteria were counted using a u.v. light microscope. The mean value was

calculated by averaging the number of bacteria present in 100 cells.

Analytical procedures

The analytical procedures were performed on eight control and eight Mg-deficient rats except for Mg determination in PMN. In order to obtain a sufficient amount of PMN for Mg analysis, PMN from four rats were pooled to obtain six samples for each group before atomic absorption spectrometric analysis. The number of total white cells was determined by a cell counter (Minos Vet, ABX, Montpellier, France). The differential leucocyte counts for polymorphonuclear cells were made from a blood smear stained with the May-Grünwald and Giemsa stain (Sigma, St-Quentin-Fallavier, France). Plasma interleukin 6 concentrations were measured by using bioassays with L929, a fibroblast cell line and B9, a murine hybridoma cell line (Givalois *et al.* 1994). NO was measured in deproteinized plasma of control and Mg-deficient rats as previously described (Rock *et al.* 1995*a*). For lipid peroxidation studies of heart, tissue homogenates were prepared on ice (1 g wet tissue + 9 ml 150 mM-KCl) using a Polytron homogenizer (Kinematica GMBH, Luzern, Switzerland). Thiobarbituric acid-reactive substances were determined in butylated hydroxy toluene-free tissue homogenates after lipid peroxidation induced by $FeSO_4$ (10 mM)–ascorbate (250 mM) for 30 min in a 37°C water bath in an O_2-free medium as previously described (Rayssiguier *et al.* 1993). Flame atomic absorption spectrometry (Perkin Elmer 800; Perkin Elmer, St Quentin en Yvelines, France) was used for plasma Mg concentration and Mg content of PMN (Gueux *et al.* 2001). Ionized plasma Mg was determined by using an AVL 977/4 analyser (AVL Medical Instruments, Eragny, France).

Statistical analysis

Statistical analysis was conducted using InStat package (Instat; Graph Pad Inc., San Diego, CA, USA). Results were expressed as means values with their standard errors. The statistical significance of differences between means was made by Student's *t* test or paired Student's *t* test. When error variance was found to be heterogeneous, the statistical significance between groups was made by using Mann-Whitney test. The differences were considered to be statistically significant when *P* value was less than 0·05.

Results

The classical signs of Mg deficiency including growth retardation were observed in Mg-deficient rats. Decreased plasma total and ionized Mg levels indicated that the rats fed the Mg-deficient diet were indeed Mg deficient (Table 1). Hypomagnesaemia was accompanied by significant increase in blood Ca concentration. Peripheral vasodilation with hyperaemia of the ears was apparent in all Mg-deficient rats. Spleen weight was significantly higher for Mg-deficient rats compared with controls. The inflammatory response was associated with a marked increase in the number of blood leucocytes and PMN (Table 1). Higher plasma interleukin 6 levels were found in Mg-deficient rats compared with control rats and plasma NO concentrations were increased in Mg-deficient animals as compared with controls. Mg deficiency increased lipid peroxidation in the heart as shown by thiobarbituric acid-reactive substance measurements (Table 1).

Fig. 1 represents the chemiluminescence activity of isolated neutrophils from control and Mg-deficient rats. As expected, neutrophils from control rats displayed a low basal activity. Basal neutrophil activity from Mg-deficient rats was significantly elevated (about 8-fold) when compared with control rats. Neutrophils from control and Mg-deficient rats were responsive to PMA and opsonized zymosan. However, the chemiluminescence activity of PMN from Mg-deficient rats was significantly higher than cells from control rats following PMA or opsonized zymosan activation. Phagocytosis study indicated an

Table 1. Characteristics of rats fed either the control or the magnesium-deficient diet†‡

(Mean values with their standard errors)

	Control		Mg-deficient	
	Mean	SE	Mean	SE
Body weight (g)	109	2	97**	2
Plasma Mg (mmol/l)	0·82	0·01	0·17***	0·03
Plasma Ca (mmol/l)	2·64	0·03	2·86**	0·06
Plasma ionized Mg (mmol/l)	0·49	0·01	0·18**	0·02
Relative spleen weight (g/kg body weight)	4·4	0·3	10·4***	0·9
Leucocytes (10^9 cells/l)	5·19	0·5	18·2***	2·1
PMN (10^9 cells/l)	1·3	0·2	8·4***	1·3
PMN Mg (ng Mg/10^6 cells)	52	4	64	6
Interleukin 6 (pg/ml)	33	2	116***	17
NO (μmol/l)	4·2	0·3	8·1***	0·4
Heart peroxidation (TBARS nmol/g wet wt)	256	16	350***	11

PMN, polymorphonuclear leucocytes; TBARS, thiobarbituric acid-reactive substances.
Mean values were significantly different from those of the control group: **$P<0·01$, ***$P<0·001$.
† For details of diets and procedures, see pp. 108–109.
‡ Mean values are for eight rats per group, except for the Mg content of PMN in which case the values are for six samples each pooled from four animals within each group.

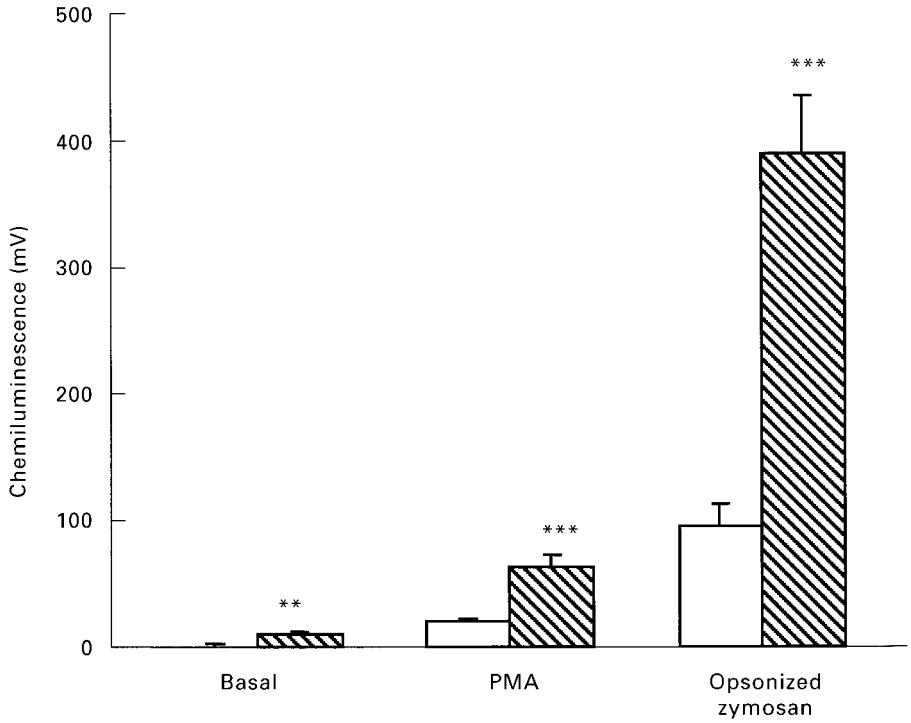

Fig. 1. Polymorphonuclear leucocyte activation in control (□) and Mg-deficient (▨) rats. Their reactive oxygen species producing activities were determined by chemiluminescence, basal or in the presence of phorbol myristate acetate (PMA) or opsonized zymosan. For details of diets and procedures, see pp. 108–109. Values are means for eight rats per group, with their standard errors represented by vertical bars. Mean values were significantly different from those of the control group: $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$.

increased capacity of PMN from Mg-deficient rats to adhere to glass (results not shown) and PMN from Mg-deficient rats showed an increased activity of phagocytosis in comparison with neutrophils from control animals (Table 2).

High concentrations of Mg (8·0 *v.* 0·8 mM) significantly decreased the chemiluminescence activity in PMN from control rats following opsonized zymosan challenge (39 (SE 10) mV *v.* 111 (SE 22) mV; *n* 5, $P<0{\cdot}05$). Similarly, increasing extracellular Mg concentration significantly decreased the chemiluminescence activity in PMN from Mg-deficient rats following PMA or opsonized zymosan activation (Table 3).

Discussion

Dietary Mg deficiency in rats gives rise after a few days to a characteristic allergy-like crisis, the first visible symptom being a peripheral vasodilation (Nishio *et al.* 1988). The inflammatory response is associated with a marked increase in total circulatory leucocytes, predominantly in the neutrophil fraction (Kurantsin-Mills *et al.* 1997; Malpuech-Brugère *et al.* 2000). Previous observations point to the spontaneous and non-infectious induction of the inflammatory process in this experimental model (Malpuech-Brugère *et al.* 1998). Leucocytosis was associated to cellular hyperplasia of cell lines in the bone marrow (McCreary *et al.* 1967) while the greater spleen size in Mg-deficient rats is believed to be due to infiltration of the spleen with PMN and macrophages (Malpuech-Brugère *et al.* 1998). Inflammatory agonists including cytokines are elevated in Mg-deficient rats (Malpuech-Brugère *et al.* 2000). The present study confirms that Mg-deficiency elevates circulatory levels of interleukin 6, this cytokine being responsible for the synthesis of acute-phase reactants

Table 2. Neutrophil phagocytosis in response to opsonized bacteria in control and magnesium-deficient rats†‡

(Mean values with their standard errors for five rats per group)

	Control		Mg-deficient	
	Mean	SE	Mean	SE
Number of intracellular bacteria/activated neutrophils	0·1	0·05	8·0*	2·2

Mean value was significantly different from that of the control group: $^{*}P<0{\cdot}05$.
† For details of diets and procedures, see pp. 108–109.
‡ Phagocytosis was evaluated by counting bacteria incorporated in neutrophils and is expressed in number of bacteria/activated cells.

Table 3. Effect of extracellular magnesium concentration on reactive oxygen species neutrophils production after activation with phorbol myristate acetate (PMA) or opsonized in magnesium-deficient rats†‡

(Mean values with their standard errors for eight rats per group)

	Extracellular Mg concentrations			
	0·8 mM		8·0 mM	
	Mean	SE	Mean	SE
PMA	76	16	54**	12
Opsonized zymosan	262	27	112**	15

† For details of diets and procedures, see pp. 108–109.
‡ Reactive oxygen species production was determined by chemiluminescence and expressed in mV.
Mean values at 0·8 mM were significantly different from those at 8·0 mM: **$P<0·01$.

(Malpuech-Brugère *et al.* 2000). The involvement of free radicals in Mg deficiency is well documented in spite of the difficulty of demonstrating their presence of effects *in vivo*. Tissues of Mg-deficient rats had a greater tendency to undergo lipid peroxidation than do tissues of control animals as shown by measurement of thiobarbituric acid-reactive substances (Rayssiguier *et al.* 1993). Other studies with a spin-trapping technique also indicated that Mg deficiency results in an increase in production of free radicals (Rock *et al.* 1995). Increased circulating levels of NO may be the result of excessive NO production by activated cells during the inflammatory response and may contribute to the increased oxidative stress due to the formation of peroxynitrites (Rock *et al.* 1995*a,b*; Mak *et al.* 1996).

Neutrophils are well known as a prominent cell type during acute inflammation and those cells find an important role through activities such as phagocytosis, production of reactive oxygen species and as a source of immunoregulatory cytokines (Cassatella, 1995). It is now recognized that respiratory burst enzymatic complex, a plasma-membrane-bound NADPH oxidase is normally dormant in resting cells. Upon several forms of cell activation, this oxidase becomes activated by a series of complex intracellular processes and reduces oxygen in one electron step to form superoxide anion, which is the primary product of the oxidative burst (Babior, 1984). The link between the external environment of the neutrophil and the activation of its function (chemotaxis, adherence, phagocytosis, oxidase activation, degranulation) is provided by the plasma membrane receptors (Edwards, 1991). Neutrophils isolated from Mg-deficient rats after 8 d on the deficient diet generate higher blood levels of superoxide anion as shown by chemiluminescence activity. Thus, PMN which are found in greater number in the blood of Mg-deficient rats are activated endogenously. As neutrophil activation is a major source of cellular oxidants, it contributes to an increase in oxidative stress in this experimental model. *In vitro*, neutrophils can respond to different agonists. PMA is a direct protein kinase C activator while opsonized zymosan gives rise to a more complex transduction signal implicating membrane receptors and internalization process (Edwards, 1991). Superoxide anion production following PMA or opsonized zymosan was higher in PMN from Mg-deficient animals as compared with control animals, i.e. these cells had an enhanced response to stimulation. This result is consistent with previous results (Malpuech-Brugère *et al.* 2000). The chemiluminescence activity of resident macrophages from Mg-deficient rats was higher compared with cells from control rats and are primed for superoxide production following PMA stimulation. In another study, a longer period of Mg-deprivation was necessary to obtain an increase in basal activity of neutrophils and upon challenge with PMA, PMN from Mg-deficient animals exhibited no significant activation, suggesting that these cells were unable to be further stimulated (Mak *et al.* 1997). By contrast, the present experiment clearly indicates that PMN activation is an early consequence of Mg deficiency and that primed PMN are able to be further stimulated by various activators. These discrepancies may be related to a cellular response that is probably different in the acute phase, as shown in the present experiment, compared with a longer period of deficiency.

The underlying mechanism for inflammatory response in Mg-deficiency remains unclear. 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 suggested (Weglicki & Phillips, 1992), but in the same experimental model as that in the present experiment, high levels of interleukin 6 were detected without a significant increase in the circulating level of substance P (Malpuech-Brugère *et al.* 2000). There is increasing evidence of the importance of a cytokine cascade in the induction and control of the inflammatory reaction. It is well known that exposure of PMN to cytokines leads to a rapid increase in expression of some plasma membrane receptors and primes the respiratory burst to generate elevated levels of oxidants (Edwards, 1991). Thus, inflammatory cytokines may upregulate neutrophil function in Mg-deficient rats. It was also suggested that NO overproduction during Mg deficiency participates in the neutrophil activation process (Mak *et al.* 1997). The present study indicates that a high extracellular Mg concentration is able to attenuate neutrophil respiratory burst in control rats. Chemiluminescence activities of PMN from Mg-deficient rats were also decreased following PMA or opsonized zymosan challenge. Since this phenomenon was observed whatever the activator used, Mg may act via a non-specific mechanism. Ca is also recognized as an important second messenger in the signalling process of neutrophil oxidative burst by stimulating protein kinase C. Increased influx of Ca from the extracellular space and release from intracellular stores are important in the oxidative burst (Snyderman & Uhing, 1992). Mg acts as a natural Ca antagonist (Iseri & French, 1984) and the possibility exists that decreased extracellular Mg increases intracellular Ca and that an increase in extracellular Mg has the opposite effect. Thus, increasing external concentration of Mg may have beneficial anti-inflammatory effects while a reduction in the extracellular Mg concentration might participate in phagocytic cell activation. The increase in plasma Ca concentration is a well-known response in Mg-deficient rats (Rayssiguier *et al.* 1982) and the possibility exists that Ca elevation participates in the phagocytic cell activation by a further

decrease in the Mg:Ca extracellular ratio. This hypothesis concerning the effect of extracellular Mg concentration on cellular response is supported by *in vivo* studies showing enhanced tumour necrosis factor-α production following endotoxin challenge in Mg-deficient rats and suggesting the inhibitory effect of high concentration of Mg on cytokine production (Malpuech-Brugère *et al.* 1999). Feeding the animals with Mg-deficient diet results in a rapid decline in plasma Mg while the Mg cellular concentration falls only slightly in muscles and soft tissues (Vormann *et al.* 1997) and do not fall at all in PMN. Plasma and interstitial body fluids represent the central compartment of Mg metabolism. This central compartment containing only 1 % total body Mg is connected to several peripheral compartments like muscles and soft tissues. Bone Mg is the most important store. However, when Mg intake is severely restricted in rapidly growing rats, renal conservation and bone mobilization are not enough to permit plasma Mg homeostasis (Classen *et al.* 1994). The decrease in plasma Mg is accompanied by a decrease in ionized plasma Mg, as shown in the present experiment. However, the percentage of ionized plasma Mg in Mg-deficient rats was increased as compared with control rats as shown previously (Zimmerman *et al.* 2000).

We have found that PMN activation is an early consequence of Mg deficiency and the exact mechanism of this effect is under investigation. Mg deficiency induces an acute-phase inflammatory response that is followed by a chronic inflammatory phase (Weglicki & Philips, 1992; Rayssiguier *et al.* 2001). However, inflammation may be switched off by inhibitory mechanisms and additional studies are needed to determine if Mg deficiency of long duration induces exhausted activity of phagocytic cells (Nishio *et al.* 2001). The inhibitory effect of supraphysiological Mg level on leucocyte activation was demonstrated *in vitro*. Further studies are needed to assess if increasing Mg concentration *in vivo* results in a decrease in leucocyte activation. Although the relevance of the findings of the present experiment to the disease process remains to be established, the results suggest the importance of the immuno-inflammatory process in the pathology of Mg deficiency. The hypothesis that Mg deficiency can lead to enhanced atherogenesis through the inflammatory process has been recently presented. Moreover, Mg deficiency amplifies myocardial vulnerability to toxic agents including ischaemia–reperfusion (Seelig, 1989). The role of PMN in ischaemia–hypoxia reperfusion damage is well documented. Migration of PMN into the ischaemic tissues, reactive oxygen species generation, release of other cytotoxic substances has been demonstrated (Sethi *et al.* 1999). Protection against ischaemia and reperfusion injury has been reported after treatment with Mg (Shibata *et al.* 1999). The results of the present experiment are consistent with the hypothesis that changes in the response of phagocytic cells resulting from an increase in plasma Mg may contribute to the protective effects of this cation.

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