The Determination of blood mononuclear Cell Magnesium content in normal Subjects

H. Millart, D. Lamiable, Ph. Collery* and H. Choisy

Zusammenfassung

Die Technik der Zelltrennung mit Verwendung von Ficoll-Hypaque wurde in dieser Studie benutzt. Das Blut von 35 gesunden Freiwilligen wurde für die Bestimmung des Magnesiumgehaltes in den mononuklearen Zellen im Plasma und in den roten Blutkörperchen herangezogen.
Die Ergebnisse (Durchschnitt ±S.D.) zeigen für die mononuklearen Zellen eine Konzentration von 88,2±15 fg/Zelle und 1,34±0,26 µg/mg Protein. Diese zwei verschiedenen Einheiten stehen in einem positiven Zusammenhang ($r=0{,}42$, $p<0{,}02$), obwohl es keinen Zusammenhang zwischen diesen Parametern und dem Magnesium im Plasma oder in den Erythrozyten gibt.
Die Proteinkonzentration der mononuklearen Zellen steht in einer negativen Wechselbeziehung zum Magnesiumgehalt dieser Zellen wenn er in µg/mg Protein angegeben wird ($r=-0{,}53$, $p<0{,}01$), aber diese Konzentration steht in einem positiven Zusammenhang mit dem Magnesiumgehalt wenn er in fg/Zelle angegeben wird ($r=0{,}54$, $p<0{,}01$).
Der Proteingehalt pro Zelle ist ein Zeichen für das Zellwachstum; die zwei Einheiten bei der Angabe der Ergebnisse ergänzen sich und müssen berücksichtigt werden.
Eine längere Untersuchung zeigt ganz klar diesen scheinbaren Widerspruch. Diese Studie zeigt, daß der Magnesiumgehalt in fg/g oder in µg/mg Protein nicht parallel verläuft.

Summary

Separation techniques employing Ficoll-Hypaque have been used for this assay. The blood from 35 normal volunteers was assayed for mononuclear cell magnesium content, plasma and erythrocyte magnesium concentrations. The results (mean +/− S.D.) showed a blood mononuclear cell magnesium of 88.2 +/− 15 gf/cell and 1.34 +/− 0.26 µg/mg protein. Regression analysis of the magnesium content of mononuclear blood cells (MBC), expressed per µg/mg protein and fg/cell, showed a significant correlation ($r=0.42$, $p<0.02$). However, by either method there was no significant correlation with the plasma or erythrocyte magnesium concentration. The protein content of MBC was negatively correlated with the magnesium content expressed per µg/mg protein ($r=-0.53$, $p<0.01$) whereas it was positively correlated when expressed per fg/cell ($r=0.54$, $p<0.01$). As the protein content per cell is a current index of cell growth the two modes of expression are complementary and need to be considered. The apparent discrepancy between these different units was well documented by a long term study where the MBC magnesium expressed either in fg/cell or in µg/mg protein did not vary parallelly.

Laboratoire de Pharmacologie, *Service de Pneumologie, Hôpital Maison Blanche, Centre Hospitalier Régional, 45, rue Cognacq-Jay, F-51092 Reims Cedex, France

Résumé

La technique de séparation des cellules mononucléées sanguines utilisant un gradient de Ficoll-Hypaque a été utilisée dans cette étude. Le sang de 35 volontaires sains a été utilisé pour le dosage du magnésium dans les cellules mononucléées, le plasma et les globules rouges.
Les résultats (moyenne +/− D.S.) montrent pour les cellules mononucléées une concentration de 88,2 +/− 15 fg/cellule et de 1,34 +/− 0,26 µg/mg de protéine. Ces deux modes d'expression des résultats sont positivement corrélés ($r=0{,}42$, $p<0{,}02$) alors qu'il n'y a aucune corrélation entre ces paramètres et le magnésium plasmatique ou érythrocytaire.
La concentration en protéines des cellules mononucléées est négativement corrélée avec le taux de magnésium de ces mêmes cellules exprimé en µg/mg de protéine ($r=-0{,}53$, $p<0{,}01$), alors qu'elle est positivement corrélée lorsque celui ci est exprimé en fg/cellule ($r=0{,}54$, $p<0{,}01$).
Le taux de protéines par cellule étant un indice de la croissance cellulaire les deux modes d'expression des résultats sont complémentaires et doivent être pris en compte. Cette apparente contradiction est bien mise en évidence par une étude prolongée qui montre que les taux de magnésium exprimés en fg/cellule ou en µg/mg de protéine n'évoluent pas parallèlement.

Introduction

The determination of plasma magnesium is the most common way to demonstrate magnesium deficiency [3, 21]. The determination of this element in erythrocytes has been used as an intracellular marker of magnesium balance. However too many parameters related to red blood cell (RBC) magnesium are involved and the real value of RBC magnesium as a clinical tool has yet to be demonstrated [14, 24]. More recently different techniques for the determination of mononuclear blood cell (MBC) magnesium content have been published and claimed to be of better value for the total body magnesium status [10, 11, 15]. However only a few clinical data are now available to evaluate those techniques [5, 6, 7, 18, 19].
Mainly two points are still to be cleared. At first the reliability of this new method, second the meaning of a contingent decrease or increase in MBC mag-

nesium. The aim of this work was to study the reliability of the technique in order to use it as a current laboratory test. We turned at first our attention to the possibility of a systematic bias related to the separation technique such as, the influence of the MBC concentration, the degree of contamination with other cellular elements. We report results for 35 normal volunteers. Among these subjects comparisons were done between MBC magnesium, plasma magnesium and RBC magnesium.

Material and Methods

Ficoll-Hypaque Separation and Magnesium Determination

Apparatus: Magnesium was determined using an atomic absorption spectrometer (Perkin, Elmer, Model 5000). A Jouan E 81 centrifuge was used to wash and separate the mononuclear cells. A Potter homogenizer was used for the cell disruption. Corex tubes (15 × 102 mm) were purchased from Corning.
Reagents: The Ficoll-Hypaque reagent was obtained from Pharmacia France. A buffered-saline glucose solution (BSG) with a pH of 7.4 and an osmolarity of 290 mosm/l was prepared at room temperature using the following analytical grade chemicals; NaCl, 8.1 g/l (138.7 mmol/l); Na_2 HPO_4, 1.22 g/l (8.6 mmol/l), NaH_2 PO_4, 0.168 g/l (1.4 mmol/l) and glucose 2.0 g/l (11.1 mmol/l). The pH was adjusted with HCl and NaOH.
Analytical Method: The separation technique was derived from the method published by *Hosseini* [15] with some modifications. Twelve milliliters of heparinized blood were mixed with 12 ml of 9 g/l NaCl. Three milliliters of Ficoll-Hypaque were pipetted into each of six Corex tubes and 4 ml of the blood, diluted with NaCl, were carefully layered over the surface of the Ficoll-Hypaque. The tubes were then centrifuged 30 min at 1 100 g at room temperature (20 – 22° C). The mononuclear cell layers were harvested using a Pasteur pipette. The mononuclear cells formed a discrete layer at the interface between the plasma saline layer (above) and the Ficoll-Hypaque (below). The mononuclear cells were pooled into one tube containing 1 ml BSG. The cell suspension was centrifuged for 15 min at 50 g at room temperature. The supernatant was discarded and the cell pellet resuspended in 10 ml of BSG. The cell suspension was gently mixed with a Pasteur pipette until a uniform suspension was achieved. Aliquots of the cell suspension were removed for a manual count using a Malassez chamber and for a differential count of white blood cells with a Coulter Counter® (Coulter Electronics). Then the cell number was adjusted to 9×10^6 cells/10 ml with the BSG solution and the cell number checked after this adjustment. 10 ml of the normalized cell suspension were then centrifuged for 10 min at 1 800 g at room temperature. The whole isolation procedure took about 90 mn. The supernatant was removed and the cell pellet lysed with 2 ml of bi-distilled water: the pellet was stirred with a Vortex and frozen (– 20° C), brought to room temperature and the suspension ground with a Potter homogenizer. To determine the magnesium concentration of the cell lysate by atomic absorption spectrophotometry, the lysate was diluted twofold with 5 g/l lanthanum oxide according to *Elin* [11]. The erythrocytes were washed three times with 0.9 % NaCl at room temperature. Red blood cells were lysed by hypotony with bidistilled water. Magnesium content in cells was measured after lysis of cells by atomic absorption spectrophotometry [17]. The protein concentration of the cell lysate was determined by the microbiuret method [16].

Application to normal Volunteers

Approximately 20 ml of blood were obtained from each volunteer by venipuncture between 8 a.m. — 9 a.m. The blood samples were taken after an overnight fast. The blood was dispensed into two Vacutainer tubes containing sodium heparin and mixed gently. 12 ml of the whole blood were used for the Ficoll-Hypaque gradient and the remainder of the sample was used to determine the plasma and erythrocyte magnesium concentrations. 35 normal volunteers were checked for magnesium concentration in plasma, erythrocytes and mononuclear cells.
Reproducibility: To assess the in vitro variability a within-run study among 8 normal volunteers was performed as following. The subjects were checked from two consecutive tubes of blood, and the Ficoll-Hypaque separations and the determinations of the above parameters performed just after the venipuncture.
To assess the in vivo variability, two studies were performed. The former was performed as Following: 6 normal volunteers were checked for MBC, plasma and RBC magnesium for three consecutive days (between run study). The latter study (long term study) was performed as following: 9 normal volunteers were checked for MBC magnesium in the middle of the following months: April, June, September, November 1985, January, March, May 1986.

Statistical Analysis

The results are expressed as the mean +/− the standard deviation (M +/− S.D.). Student's t-test was used to compare differences in populations. Linear regression was used to analyze the results and the Student's T test has been used for calculating the significance p of the correlations-coefficient r according to *Schwartz* [20].

Results

1. Influences of the blood mononuclear cell concentration and of the initial cell count after separation with the Ficoll-Hypaque discontinuous gradient on MBC magnesium. Partial correlations were calculated between those three variables. As expected the initial mononuclear cell count, either manual 12.4 +/− 3.9×10^6 cells/10 ml or with the Coulter Counter 11.4 +/− 3.6×10^6 Cell/10 ml was correlated with the MBC concentration 2.69 +/− 0.78×10^9 cells/l (P <0.02) and the yield in mononuclear cells was 36.6 +/− 10.7 %. MBC magnesium, expressed either in fg/cell or µg/mg protein was independent of the initial cell count and of the mononuclear blood cell concentration.
2. Influence of the degree of contamination with other cellular elements on MBC magnesium (Fig. 1). As shown in the Figure 1, there was a significant and negative correlation ($r = -0.48$, $P < 0.01$) between the percentage of mononuclear cells after isolation and the magnesium content expressed in fg/cell whereas no significant correlation was observed when the magnesium content was expressed in µg/mg protein.

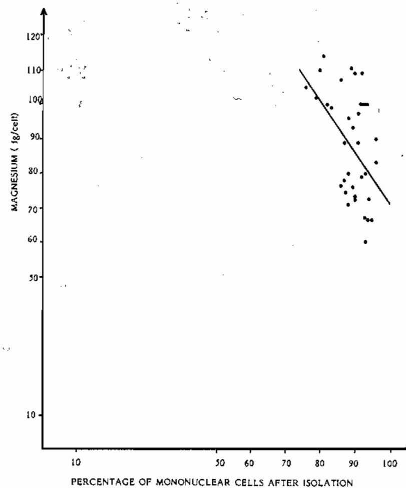


Fig. 1: Influence of the degree of contamination with other cellular elements on the magnesium content of mononuclear cells (fg/cell). The equation for the line, determined by linear regression is: $Y = -1.465 X + 218$, $Syx = 13.4$, $N = 35$

3. Reproducibility:
— The within-run study showed a coefficient of variation (CV) of 7.2 % for the initial cell count, 11.7 % for the protein determination. 9.8 % for the magnesium determination, 9.8 % for the protein content per cell, 9 % and 9.4 % for the MBC magnesium expressed in fg/cell and µg/mg protein respectively.
— The day-to-day reproducibility study ("between run") showed CVS of 2.2 % and 2.3 % for plasma and RBC magnesium respectively. The CV for MBC magnesium expressed in fg/cell was 9.05 % and 12.8 % when expressed in µg/mg protein.
— The long term study (Fig. 2) showed CVS of 20 % and 24.4 % for MBC magnesium expressed in fg/cell and µg/mg protein respectively and 28.9 % for the protein content per cell. The variations in MBC magnesium expressed in fg/cell and µg/mg protein were not parallel. The mean lower value for MBC magnesium (µg/mg protein) was observed with the higher value for the mean protein content per cell.

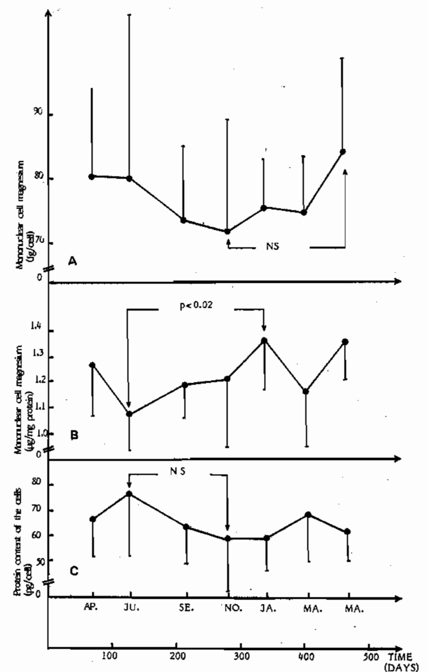


Fig. 2: Mean MBC magnesium expressed in fg/cell (mean ± S.D.) [A], in µg/mg protein [B] and Protein content of mononuclear cells [C] in nine normal volunteers. Blood samples were obtained in the middle of the following months: April (AP.), June (JU.), September (SE.), November (NO.) 1985, January (JA.), March (MA.), and May (MA.) 1986.

4. The blood magnesium values for the 35 normal volunteers are presented in table I.

There were no significant correlations for magnesium values among plasma, erythrocytes and mononuclear cells in blood. Regression analysis of the MBC magnesium, expressed per fg/cell and µg/protein, showed a significant correlation ($r = 0.424$ $p < 0.02$).

Discussion

Clinical manifestations related to magnesium deficiency are most of the time non specific symptoms. However some clinical signs can be divided into the following categories: Neuromuscular hyperactivity; cardiac arrhythmias; psychiatric disturb-

Tab. 1: Blood magnesium parameters for 35 normal volunteers

	Mean	S.D.	Range	CV, %
Age, years	33.2	4.8	24–41	14.5
Plasma magnesium, mmol/L	0.85	0.05	0.76–0.98	5.9
Erythrocyte magnesium, mmol/L	2.22	0.18	1.81–2.51	8.1
Percentage of mononuclear cell after isolation	88.6	5.0	76–96	5.6
Mononuclear cell magnesium, fg/cell	88.2	15.0	60.3–114	17
Mononuclear cell magnesium, μg/mg protein	1.34	0.26	0.94–2.09	19.4
Protein per cell (pg/cell)	67.3	13	45.7–95.6	19.3

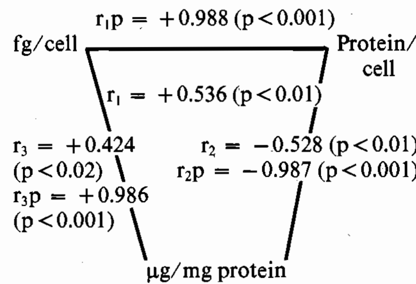
ances; potassium deficiency [3]. The multiplicity of these manifestations results in confusion and controversy. The diagnosis of magnesium deficiency can be made objectively by finding significant hypomagnesemia in a patient with an illness known to cause magnesium deficiency; however there are many exceptions where magnesium deficiency does exist with normal or elevated plasma magnesium levels [4, 8].

The evaluation of the magnesium content of peripheral mononuclear blood cells might be an index of the muscle and cardiac magnesium pool [6, 9, 12, 22] whereas serum magnesium was found to correlate closely with bone magnesium stores [2]. Simultaneous determination of these two parameters could be of better value than the classic determination of plasma and erythrocyte magnesium concentrations. There is a need clinically for a better assessment of intracellular magnesium and/or magnesium balance. The estimation of intracellular magnesium concentration by means of the RBC magnesium is of limited value at least for three reasons: first the erythrocyte is unprovided with nucleus and mitochondria, second RBC magnesium is a function of red cell turnover [24], third the erythrocyte pool contains approximately 0.5 % of the total body magnesium [15].

A preliminary study (unpublished data) evidenced a negative and significant correlation ($r = -0.54$, $p < 0.01$, $N = 23$) between the initial cell count after isolation and the magnesium content per cell. This correlation was no longer observed after the adjustment procedure. Since the measurements were made on 9×10^6 cells per 10 ml the influence of any variation in the cell number for magnesium analysis would be minimized. We counted the number of cells in the original cell suspension and the number of cells after the adjustment to 9×10^6 cells/10 ml with the BSG solution. The percent recovery was 99.8 % with a CV of 5.7 %. The centrifugation after the adjustment of 9×10^6 cells per 10 ml caused a partial lysis of mononuclear cells as shown by the presence of Mg and proteins in the supernatant BSG. The precision of the ratio Mg in the supernatant/Mg in the pellet and the ration proteins in the supernatant/proteins in the pellet has been evaluated by means of the CV and was 19.6 % and 19.7 % respectively. No cells could be seen in the supernatant BSG under microscopic observation. Centrifugation may affect cell magnesium because excessive acceleration forces cause fragile cells to lyse. The magnesium found in the supernatant BSG may originate from magnesium in the solution surrounding the cells, transport of magnesium across the cell membrane, or lysis of mononuclear cells during the washing procedure. However insufficient acceleration forces do not pellet larger, younger cells. Working with the cells at room temperature is necessary in order to avoid an important drop of the cationic content of the cells. Usually the final centrifugation is performed at 2 000 g for 10 mn [10, 11, 15]. *Elin* [10] found in the supernatant BSG from the cell pellet a magnesium concentration of 8 μEq/l whereas in our experimental conditions we found a concentration (Mean +/− S.D.) of 2.7 +/− 0.5 μEq/l.

The degree of contamination with platelets, erythrocytes and granulocytes was checked after each isolation procedure. Platelet contamination was not observed in the current study and erythrocyte contamination of the mononuclear cell population was very low (mean +/− S.D.) 0.015 +/− 0.008 %. As the approximate platelet magnesium content has been reported to be 3 fg/cell and erythrocyte magnesium content to be 5 fg/cell [15], minor contamination of the blood mononuclear cell population with platelets and erythrocytes should not significantly affect the mean blood mononuclear cell magnesium content. The negative correlation observed between MBC magnesium (fg/cell) and the percentage of mononuclear cells after isolation is probably due to granulocyte contamination which contains more magnesium per cell [11]. The fact that granulocytes have a larger size than do mononuclear cells could explain their higher content in magnesium per cell. It is noteworthy that the mononuclear magne-

sium concentration per protein was not correlated with the granulocyte contamination.
Therefore it would be desirable in routine analysis to mention in the results the degree of contamination and if too high the analysis should be performed again.
The choice of the accurate unit for expressing the magnesium content of mononuclear blood cells deserves some discussion. Several different units have been chosen for the denominator for expressing cellular magnesium: mmol per kg dry weight [6, 12, 17], nmol or μg/mg protein [13, 19], pmol/100 cells [1], fg/cell and μg/mg protein [10, 15] or fg/cell and per unit weight of DNA [11].
As shown in the within-run study the cell count had the lowest CV among all the variables studied. *Elin* [11] prefers to express the results per unit weight of DNA rather than in fg/cell. However this preference is not convincing when comparing the respective interindividual CVs (19.9 % VS 20 %). The interindividual CV related to the expression of our results in μg/mg protein (19.2 %) was comparable in our study to the CV related to the expression of the results in fg/cell (17.1 %).
Protein assay with the lowry method cannot be used with the separation medium [15]. The micro-biuret method has been used in our study and no interferences were observed with the Ficoll-Hypaque reagent; furthermore no bovine serum albumin was added during the separation procedure, to avoid protein contamination.
As shown in the results a weak but significant correlation was observed in our work between the two units used for the evaluation of MBC magnesium. This weak correlation was not due to a defect in the protein determination but to a third variable i.e. the protein content per cell. Calculation of the partial correlations between the three parameters (fg/cell, μg/mg protein and protein per cell) showed that when the protein per cell was considered as a constant, the partial correlation (rp) between the MBC magnesium (fg/cell) and the MBC magnesium (μg/mg protein) was highly significant ($r_3p = 0.986$, $p < 0.001$).
The following diagram summarizes all the correlations between the three variables.



As the protein content per cell is a current index of cell growth and cell size it can be conceivable that the total amount of magnesium will increase with the size of the cell: it does not mean necessarily an increase in magnesium per volume of cell. Magnesium is present at a regulatory concentration for protein synthesis; it is plausible that magnesium plays a role in the regulation of protein synthesis and therefore cell growth [23]: this might explain the negative correlation observed between the MBC magnesium (μg/mg protein) and the protein content per cell. This hypothesis deduced from static data seems to be confirmed by the long term or dynamic study (Fig. 2).
In other words the two ways for expressing the magnesium content of mononuclear blood cells are complementary and not mutually exclusive. It might be hazardous to ignore one of them and to give a verdict of magnesium depletion.
The interindividual CV for the MBC magnesium (fg/cell and μg/mg protein) was higher than the ones for plasma and RBC magnesium. One of the most significant factors for the high CV is the method itself as shown in the present within-run study. Besides the degree of contamination with other cellular elements varies from preparation to preparation and among individuals.
The interindividual CV of 17.1 % was of the same order as the one (20 %) published by *Elin* [11], when the results were expressed in fg/cell. The variations observed with regard to the protein content per cell was probably an other cause of the high interindividual CV. An other parameter involved in the high interindividual CV might be the presence of lymphocyte subpopulations.
The interindividual CV for the protein content per cell was 19.3 %: if we assume that this parameter is a function of cell growth and therefore linked to the mean cell age of mononuclear blood cells, it seems improbable that the intraindividual variability could be less for a long period except for a short time study as reported by *Elin* [11] and in our between-run study. This is confirmed by the high mean CV (28.9 %) observed for this parameter in our long term study. A question raises from this remark: is it possible to affirm magnesium deficiency by a simple determination of MBC magnesium and an unique expression of the results in fg/cell or in mg/g of DNA which is about the same for diploid cells with a low turnover. The expression of the results with the simultaneous use of the units μg/mg protein and fg/cell probably brings more informations with regard to the intracellular magnesium concentration than the simultaneous use of the units fg/cell and mg/g of DNA which brings more accuracy.

In conclusion the determination of MBC magnesium does not require sophisticated equipments. Using our method a laboratory assistant can perform four determinations a day. For the moment one must be cautious because, though nucleated cells, blood mononuclear cells represent only a small compartment of the total body magnesium, factors other than the status of the total body magnesium such as cell age could appreciably change the magnesium content of mononuclear blood cells, and the degree of contamination with other cellular elements can change the results.

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For the authors: *Hervé Millart, M. D.*, Laboratoire de Pharmacologie, Hôpital Maison Blanche, Centre Hospitalier Régional, 45, rue Cognacq-Jay, F-51092 Reims Cedex (France)