Intracellular and extracellular blood magnesium fractions in hemodialysis patients; is the ionized fraction a measure of magnesium excess?

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To establish the best measure for determining magnesium overload, we measured ionized and total magnesium in serum and mononuclear blood cells and total magnesium in erythrocytes in blood of 23 hemodialysis patients, known for their disturbed magnesium homeostasis. When comparing the mean magnesium values obtained in the patient population with those of a control population, all of these magnesium markers, including the biologically active fractions, were significantly (P < 0.05) increased. Because serum total magnesium was not increased in all dialysis patients studied, the population was divided into two groups, according to total serum magnesium >1.0 mmol/L or less than that. Results in these two populations showed that ionized serum magnesium and ionized magnesium in mononuclear blood cells might give a better indication about the magnesium status of the tested patients than the currently used total serum magnesium data. However, neither of the two markers, especially ionized serum magnesium, was able to discriminate fully between normal magnesium homeostasis and magnesium excess. We therefore conclude that the two ionized magnesium markers offer minimal advantage for this discrimination, and that the total magnesium concentration in serum remains the measurement of choice.

In trying to obtain information about the magnesium (Mg) status of a patient, clinician and clinical chemists are generally confined to measuring the total magnesium concentration in serum (tMg)s. However, around 1990, ion-selective electrodes for determining ionized Mg in blood became available [1]. In our laboratory we evaluated one of these electrodes and subsequently established reference values with it [2, 3]. This method measures the biologically active Mg fraction, which possibly gives more reliable information on the functional Mg status. However, because serum Mg represents <1% of total body Mg, several techniques for measuring intracellular Mg in many kinds of tissues and cells have been published [4–6].

In 1991, Ng et al. described a method for measuring ionized Mg in human lymphocytes, using a Mg-sensitive fluorescent probe in combination with spectrophotometry [7]. We tested several aspects of this so-called multiple-cell detection method (MCDM) and compared the results by this system with the intracellular ionized Mg concentrations measured by flow cytometry, a so-called single-cell detection method (SCDM). We concluded (see Appendix) that Mg concentrations measured by both techniques are comparable, but we prefer MCDM because it is more easily accessible and less expensive, even though flow cytometry has the advantage of detecting single cells.

In the present study we tried to assess which Mg marker in blood might best indicate deviations from the “normal” condition in healthy subjects. Therefore, we measured extracellular Mg and intracellular Mg, both ionized concentrations and total concentrations, in blood of 23 hemodialysis patients. Known for their disturbed

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Nonstandard abbreviations: RBC, erythrocytes; MBC, mononuclear blood cells; tMg, total serum magnesium; iMg, ionized serum magnesium; tMgRBC, total magnesium in RBC; tMgMBC, total magnesium in MBC; iMgMBC, ionized magnesium in MBC; iMgL, ionized magnesium in lymphocytes; SCDM, single-cell detection method; MCDM, multiple-cell detection method; FSC, forward scatter; SSC, sideway scatter; AM, acetoxymethyl ester; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid.
Mg homeostasis [5], these patients represent a model for hypermagnesemia in this study. We then compared the intracellular and extracellular Mg fractions in this specific patient population with the commonly used tMg₆ value and with Mg values measured in blood from healthy volunteers.

Materials and Methods

SUBJECTS
Venous blood used for measurements of ionized and total Mg in serum was drawn in plain, nonsiliconized 4.5-mL tubes. Blood for determining total Mg concentrations in mononuclear blood cells (MBC; tMgMBC) and in erythrocytes (RBC; tMgRBC) was collected in 10-mL heparin-containing tubes, and blood for determining ionized Mg in MBC (iMg²⁺MBC) was drawn into 10-mL tubes containing 0.8 g of polystyrene granules. All tubes used were Vacutainer Tubes (Becton Dickinson).

Reference values for iMg²⁺MBC were obtained by drawing blood from 40 healthy laboratory workers (16 men, 24 women; median age 36 years, range 21–54 years). Reference values for the other Mg markers had already been established during previous Mg studies in our laboratory [3].

The chronic hemodialysis patients (16 men, 7 women; median age 60 years, range 26–85 years) participating in the study were in a stable condition, hydrated to normal on clinical grounds, and had been treated with hemodi-

lation for a median of 15 months (range 3–188 months).

All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

CHEMICALS
Fluorescent probes [Mag-indo-1/acetoxymethyl ester (AM) and Mag-fura-2/AM] were obtained from Molecular Probes Europe. The ionophore 4-Br-A23187 and nigericin were obtained from Sigma Chemical Co., EDTA and ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetra-

cetic acid (EGTA) from Boehringer Mannheim GmbH. Other chemicals, all of analytical-reagent grade, were purchased from E. Merck BV.

PROCEDURES
Measurement of total and ionized Mg in serum. After clotting (45 min) and centrifugation (10 min, 1500g), serum was separated from the cells and stored in completely filled rubber-sealed air-tight tubes at −20 °C, for no more than 4 weeks. After thawing, the pH was only slightly increased, so the ionized Mg fraction did not change significantly. tMg₆ was measured by atomic absorption spec-

troscopy (PE2100; Perkin-Elmer), the ionized serum Mg (iMg²⁺) by a Mg ion-selective electrode installed in the Microlyte 6 ion analyzer (KONE Instruments).

Isolation of MBC and loading with fluorescent probe. MBC and RBC were used for the determination of the total and ionized intracellular Mg concentration were isolated from hepa-
rinized and defibrinated blood, respectively, as described previously [8]. After counting and differentiating the cells, we dissolved in 1.0 mL of H₂O the isolated MBC used for the determination of tMgMBC; for iMg²⁺MBC determinations, we dissolved the cells in 2.0 mL of buffer containing (in mmol/L): 94.1 NaCl, 23.8 NaHCO₃, 5.64 Na₂HPO₄, 0.42 Ca(NO₃)₂, 5.36 KCl, 0.41 MgSO₄, 11.1 glucose, and 25 HEPES, pH 7.35, plus 50 mL/L fetal calf serum.

We used two different fluorescent probes, loading the cells with Mag-indo-1/AM or Mag-fura-2/AM at final concentrations of 10.9 and 9.4 μmol/L, respectively, by a modification of the method of Raju et al. [9]. The cell suspension also contained Pluronic® 20% (in dimethyl sulfoxide) and probenecid at final concentrations of 0.3 g/L and 1.0 mmol/L, respectively. The main difference between the two fluorescent probes is their difference in emission and excitation behavior. Mag-indo-1 is a dual-emission probe, whereas Mag-fura-2 is a dual-excitation probe. The advantage of the latter is its greater increasing ratio from minimum to maximum Mg concentration, which theoretically allows better precision of the Mg measurement. So, after comparing flow-cytometric intracellular Mg measurements with multiple-cell detection (see Appendix), we used the MCDM with Mag-fura-2 for further measurements.

Measurement of total intracellular Mg concentration. The total Mg concentration in MBC and RBC was measured as described previously [8]. The number of isolated cells was counted with a Bayer-H3 system, the protein concentration of the MBC lysate was measured photometrically after treatment with Coomassie Brilliant Blue (Microprot; Oxford Labware), and Mg was measured by atomic absorption spectrophotometry, as above. The intracellular Mg concentration of MBC was expressed as μmol/g protein; that of RBC, as fmol/cell.

Measurement of intracellular ionized Mg concentration. The intracellular ionized Mg fraction was determined by using the equation established by Gryniewicz et al. [10]:

\[ iMg^{2+}_{MB} = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_i}{S_{0i}} \]  

(1)

with \( K_d \) the dissociation constant of the probe/Mg complex, \( R \) the excitation or emission ratio at the current intracellular Mg concentration, \( R_{min} \) the excitation or emission ratio at minimal intracellular Mg concentration, \( R_{max} \) the ratio at maximal intracellular Mg concentration, \( S_i \) the fluorescence intensity of the highest wavelength at
R_{min}$ and $S_b$ the fluorescence intensity of the highest wavelength at $R_{max}$. After making the cells permeable for Mg by adding the ionophore 4Br-A23187 (final concentration 7.5 μmol/L) and nigericin (final concentration 10 μmol/L), we determined $R_{max}$ through addition of EDTA/EGTA (final concentration 1.25 mmol/L each) and determined $R_{min}$ by stepwise addition of increasing concentrations of MgCl$_2$ (final concentration ~6.5 mmol/L ionized Mg). Because it is impossible to bind all Mg by EDTA/EGTA and saturate all Mag-indo-1 or Mag-fura-2, values for $R_{min}$ and $R_{max}$ were approximated by calculations based on the measured ratios, after which iMg$_2$ was calculated by use of Eq. 1.

Measurements were performed in a 2.0-mL cell suspension (0.3 × 10$^6$ to 0.8 × 10$^6$ cells/mL) in a 3.0-mL quartz cuvette at 37 °C with a dual excitation and emission spectrophotometer (SLM-Aminco, Aminco Bowman Series 2 luminescence spectrometer). Excitation was at 382 nm (free Mag-fura-2) and 345 nm (bound Mag-fura-2); emission was monitored at 496 nm. $K_d$, determined by measuring R after the addition of small amounts of MgCl$_2$ (as described in the Appendix), was found to be 1.44 mmol/L.

**Statistics**

All statistical analyses were performed with the statistical package Statgraphics (PLUS*WARE Products). Mg concentrations in different populations were compared by using the two-sample t-test, or the Mann–Whitney U-test if necessary. A two-tailed probability of $P <0.05$ was considered significant. Correlations between different Mg markers were investigated by the Kendall rank correlation test.

**Results**

**Comparison of Mag-indo-1 and Mag-fura-2**

Two different fluorescent probes were used for determining iMg$_2$ in the patient population as well as in the group of healthy volunteers. Before pooling the Mg concentrations obtained with both probes, we compared the different values by the two-sample t-test. In neither population, healthy volunteers or hemodialysis patients, was a difference detected between iMg$_2$ measured by Mag-indo-1 and by Mag-fura-2 (hemodialysis patients $P = 0.270$, healthy volunteers $P = 0.490$).

**Mg in Hemodialysis Patients and Healthy Volunteers**

Table 1 presents the mean total and ionized Mg concentrations in serum and MBC, and the total Mg concentrations in RBC of healthy volunteers and 23 hemodialysis patients. The ranges (mean ± 2 SD) of the intracellular Mg concentrations were much wider than those for Mg in serum. Nevertheless, all Mg markers, including the intracellular fractions, of the patient population were significantly greater than those of the healthy population. tMg$_{MBC}$ showed the largest increase, namely, 1.5 times the mean value for the healthy volunteers.

There was a correlation between tMg$_s$ and iMg$_2$, but also between the two serum Mg markers and tMg$_{MBC}$ in the blood of the hemodialysis patients. The Kendall rank correlation coefficient ($\tau$) was 0.517 ($P = 0.0003$) for tMg$_s$ vs tMg$_{MBC}$ and 0.352 ($P = 0.0138$) for iMg$_2$ vs tMg$_{MBC}$. No correlation was detected between iMg$_2$ and iMg$_2$ or between the other Mg markers.

We divided the hemodialysis patient group into two populations, based on whether the tMg$_s$ concentration was below or above the upper reference limit of 1.0 mmol/L. Table 2 presents the blood Mg values for each group. Treatment, duration, and frequency of dialysis of both populations were comparable. With respect to the serum Mg markers and tMg$_{MBC}$, the mean values differed significantly ($P \leq 0.05$) between the two populations, but not so for tMg$_{MBC}$ and iMg$_2$.

When comparing hemodialysis group 1 (tMg$_s$ concentration ≥ 1.0 mmol/L) with the healthy population, all intracellular Mg measures were significantly greater in the patient group, despite both groups’ having the same

| Table 1. Mg markers measured in blood of healthy volunteers and hemodialysis patients. |
|------------------------------------------|------------------------------------------|------------------------------------------|
| **Extracellular**                         | **Intracellular**                         | **MBC**                                  |
| n                                        | Mean                                     | Range                                    | Mean                                     | Range                                    |
| 81                                       | 0.88                                     | 0.75–1.01                                | 68                                       | 40                                       | 83                                       |
| 81                                       | 0.56                                     | 0.47–0.65                                | 47.5                                     | 0.24                                     | 0.18                                     |
| **Patients (n = 23)**                     | **1.01**                                 | **0.47–0.65**                            | **27.1–67.8**                             | **0.16–0.36**                             | **0.12–0.24**                            |
| Mean$^a$                                 | 1.12                                     | 0.71                                     | 53.9                                     | 0.32                                     | 0.27                                     |
| Range$^a$                                | 0.62–1.62                                | 0.47–0.95                                | 26.7–81.2                                | 0.19–0.53                                | 0.19–0.35                                |
| Ratio$^c$                                 | 1.27                                     | 1.27                                     | 1.13                                     | 1.33                                     | 1.50                                     |

$^a$ Mean ± 2 SD.

$^b$ All significantly different (two-sample t-test) from the means for the healthy volunteers. For iMg$_2$ the two-sample t-test was performed after logarithmic transformation.

$^c$ Mean for hemodialysis patients divided by mean for healthy volunteers.
The intracellular Mg 2⁺ concentration of this patient group differed from normal also ($P < 0.038$), but not as significantly as the intracellular Mg fractions (for $tMg_{MBC}$, $P = 0.003$; for $tMg_{RBC}$, $P < 0.001$; and for $iMg_{2⁺_{MBC}}$, $P = 0.003$). In hemodialysis group 2 ($tMgs = 1.0 \text{ mmol/L}$), all Mg markers except $tMg_{MBC}$ were increased in comparison with the healthy population.

**Discussion**

**COMPARISON OF MAG-INDO-1 AND MAG-FURA-2**

The measurements performed with Mag-indo-1 and Mag-fura-2 in both the healthy volunteers and the hemodialysis patients lead us to conclude that these fluorescent probes measure comparable intracellular Mg concentrations. This conclusion is supported by preceding studies with two calcium probes, Indo-1 and Fura-2, which also detected no difference [11, 12]. For the present study, therefore, we pooled the $iMg_{2⁺_{MBC}}$ concentrations measured with both probes.

**Table 2. Differences in Mg markers in blood of the two hemodialysis patient groups.**

<table>
<thead>
<tr>
<th></th>
<th>Extracellular</th>
<th>Intragranular</th>
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<tbody>
<tr>
<td></td>
<td>$tMg_{s}$, mmol/L</td>
<td>$iMg_{2⁺_{s}}$, mmol/L</td>
</tr>
<tr>
<td>Group 1 ($tMgs ≤ 1.0 \text{ mmol/L}$), $n = 9$</td>
<td>Mean 0.88 0.60$^d$</td>
<td>58.9$^d$</td>
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<tr>
<td></td>
<td>Range 0.72–1.04 0.46–0.74</td>
<td>39.2–78.7</td>
</tr>
<tr>
<td>Group 2 ($tMgs &gt; 1.0 \text{ mmol/L}$), $n = 14$</td>
<td>Mean 1.28$^e$ 0.78$^d$</td>
<td>50.7</td>
</tr>
<tr>
<td></td>
<td>Range 0.92–1.64 0.60–0.96</td>
<td>20.7–80.7</td>
</tr>
</tbody>
</table>

Range is defined as mean ± 2 SD.

$^a$ Statistical comparison of both populations by *two-sample t-test, $^b$ two-sample t-test after logarithmic transformation, and $^c$ Mann–Whitney U-test.

$^d$ Significantly different from the mean Mg value of the healthy subjects.

$^e$ Significantly different from the mean Mg value of group 1.

**MG IN HEMODIALYSIS PATIENTS AND HEALTHY VOLUNTEERS**

The human body contains $\sim 1 \text{ mol of Mg, } <1\%$ of which is present in blood. It is important to determine, therefore, which of the proposed Mg markers gives the best information about the Mg status of the body. Must clinical chemists continue reporting $tMgs$; should $iMg_{2⁺_{s}}$ become the measurement of choice; or is none of the current measurable serum markers appropriate? If none of these, might intracellular Mg markers give proper information about hyper- and hypomagnesemia (possibly depending on the type of disease)? In attempts to answer this question, several studies about measurement and correlations between Mg in serum, RBC, MBC, and tissues (e.g., skeletal muscle, heart, and bone) have been performed. Several reviews on this subject also have been published [13–15]. Although the collective results are still contradictory, to date $tMg_{MBC}$ seems to give the most appropriate estimate of intracellular Mg. In this study we focused on measurements in serum and blood cells, and evaluated the value of two new Mg markers ($iMg_{2⁺_{s}}$ and $iMg_{2⁺_{MBC}}$) for establishing whether Mg excess was present. We measured these two Mg markers and the total Mg concentration in serum, MBC, and RBC in blood of chronic hemodialysis patients. Because the kidneys play a major role in Mg homeostasis [16], chronic hemodialysis patients are often hypermagnesemic, the extent depending on the Mg concentration in the dialysis solution applied. Because of their Mg overload, these patients form a suitable model for this study [5, 17].

In comparison with the mean Mg values measured in the healthy population, all Mg markers in patients, including the biologically active fractions, were greater, but not all ratios of the Mg markers between the two groups were equal (Table 1). Only the increases in the two serum markers were exactly the same (1.27). This means that in this patient population, $iMg_{2⁺_{s}}$, the biologically active fraction, is not kept at a more constant value than $tMg_{s}$, and the two fluctuate in the same manner. Moreover, even if an active mechanism such as transmembrane Mg channels in MBC might exist to keep the ionized Mg
concentration in the cell within certain limits, it does not work properly in this patient group. The ionized fraction in MBC is even further increased than the total intracellular concentration in this type of blood cells. Remarkably, the mean iMg$^{2+}$MBC concentration in patient group 2 (tMg$>_1$1.0 mmol/L) was comparable with that in patient group 1 (tMg$<=1.0$ mmol/L) (Table 2). Therefore, the increase of iMg$^{2+}$MBC concentration seems to be restricted. No correlation between the increase of both ionized intracellular and extracellular Mg (by 33% and 27%, respectively) was noticed. This finding is supported by Niemela et al. [18], who measured ionized Mg in platelets of healthy adult volunteers and compared these values with several variables, including tMg$>_s$ and iMg$^{2+}$s.

Mag-fura has been used in two other studies to measure ionized Mg in blood cells of patients with renal insufficiency. Kisters et al. [19] determined cytosolic free Mg in lymphocytes of 12 patients with renal insufficiency and compared these values with those of 15 controls. They demonstrated that tMg$>_s$ and iMg$^{2+}$s concentrations were greater in the patient population, whereas the concentration of ionized Mg in lymphocytes was comparable in both groups. Kaupke et al. [20] measured the intracellular ionized fraction in platelets of 9 patients with end-stage renal disease and an increased tMg$>_s$, who were treated with hemodialysis (dialysate containing 0.5 mmol/L Mg). Surprisingly, the Mg concentration measured in these patients was considerably lower than in the normal group. This finding was explained as probably resulting from various clinical conditions to which patients with end-stage renal disease are particularly prone. The discrepancies between our results and those of Kisters et al. [19] and Kaupke et al. [20] show that the final word on this subject has not yet been spoken.

Because not all dialysis patients studied had increased serum total Mg, we looked at dividing the population into two groups (Table 2), according to their tMg$>_s$ concentration (cutoff value 1.0 mmol/L). Assuming that all of the patients, including those in group 1 (tMg$<=1.0$ mmol/L), had a Mg excess, we could conclude that iMg$^{2+}$s and the three intracellular Mg markers give more reliable information about the Mg status of the tested patients than does the currently used total serum Mg data (Fig. 1). On the other hand, despite the difference between the mean values of iMg$^{2+}$s, which was significant, the measured concentrations overlapped considerably, the mean iMg$^{2+}$s concentration in the group 1 hemodialysis patients falling within the 2 SD range of the control (healthy volunteer) population. This finding, including an identical increase of iMg$^{2+}$s and tMg$>_s$ seen in comparing hemodialysis patients with the control population, minimizes the clinical usefulness of this Mg measurement as a marker of a Mg excess.

Of the three intracellular markers, iMg$^{2+}$MBC and tMg$^{RBC}$deviated more from the reference value than did tMg$^{MBC}$. Even in patient group 2 (tMg$>_1$1.0 mmol/L), tMg$^{RBC}$ did not differ significantly from that seen in the control population, probably as a result of the high CV for this Mg assay (14%) [8]. tMg$^{RBC}$ values in this specific patient population result from several influences. In uremic patients RBC have a decreased life span, which results in increased erythropoiesis. Because young RBC contain much more Mg than older cells, hemodialysis patients can be expected to have an increased tMg$^{RBC}$ [21–23]. Moreover, the increased serum Mg concentration during erythropoiesis can enhance this effect. In our study, tMg$^{RBC}$ correlated with extracellular Mg (tMg$>_s$ and iMg$^{2+}$s). On the other hand, an increased rate of Na/Mg antiport in hemodialysis patients, which leads only to Mg efflux out of RBC, partially compensates for the intracellular increase [24]. In our opinion, all these influences combine to make RBC Mg an unsuitable measure of Mg overload in hemodialysis patients.

The remaining intracellular Mg marker, iMg$^{2+}$MBC, is not able to discriminate fully between normal Mg homeostasis and Mg excess (Fig. 1). Although this measurement showed the largest increase in the hemodialysis population (Table 1), and its mean concentration in patient group 1 was significantly increased (Table 2), the mean ± 2 SD concentration range for this patient group enclosed the reference interval for the control population. Therefore, although the two ionized Mg measures, iMg$^{2+}$s and iMg$^{2+}$MBC, seem to be better indicators in establishing the Mg overload in hemodialysis patients than is the regular measured tMg$>_s$ concentration, the ideal marker has yet to be found. Moreover, because the measurement of iMg$^{2+}$MBC is rather complicated, this intracellular Mg marker is not really suitable as a routine laboratory test.

We therefore conclude that in discriminating between normal Mg homeostasis and Mg excess, the two ionized Mg markers offer minimal advantage; rather, the total Mg concentration in serum remains the marker of choice in hemodialysis patients.

We thank KONE Instruments for providing us with the Microlyte 6 ion-selective analyzer and its consumables and for technical support.

Appendix: Flow Cytometry and Fluorometry Compared for Measuring Intracellular iMg$^{2+}$ in Lymphocytes

Before a final decision can be made on which magnesium fraction should be determined to monitor the true magnesium status of the body, it is necessary to investigate which method is preferable for determining ionized intracellular Mg. Here we discuss which method is preferable

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4 The work reported here was performed by Renata Sanders, Marjolein G. Klous, Henk J. Huijgen, Rudolf W. van Olden, and Gerard T.B. Sanders, with grateful appreciation for the technical assistance of Ron Hoebe and Carel van Oven of the Department of Radiobiology of the University of Amsterdam. Address correspondence about the Appendix to R.S. Fax 31-20-5664440.
for determination of ionized magnesium in lymphocytes (iMg\textsuperscript{2+}{\textsubscript{L}}), flow cytometry or fluorometry.

Some studies have already demonstrated determination of iMg\textsuperscript{2+}{\textsubscript{L}} in a cell suspension with use of a magnesium-sensitive fluorescent probe and a fluorometer [6, 19, 25–27]. In this multiple-cell detection method (MCDM) setup, the result for iMg\textsuperscript{2+}{\textsubscript{L}} is strongly affected by the use of an extracellular probe that allows the analyte to leak out of the cells into the surrounding buffer. Avoiding this interference requires a single-cell detection method (SCDM), such as confocal laser scanning microscopy or flow cytometry. Because the flow cytometer is now a common tool in clinical chemistry, we chose to use a flow cytometer equipped with a UV laser for this comparison study.

Using a SCDM to determine the iMg\textsuperscript{2+}{\textsubscript{L}} concentration has other advantages: Because iMg\textsuperscript{2+}{\textsubscript{L}} is determined in each individual cell, we can study the distribution of iMg\textsuperscript{2+}{\textsubscript{L}} in a sample [28]; moreover, a certain population of cells can be selected, which means that the purity of the initial sample (isolation of lymphocytes from other MBC) is less important.

In this study, we wanted to determine the preferred method for measuring iMg\textsuperscript{2+}{\textsubscript{L}} by comparing the SCDM and the MCDM (flow cytometry vs fluorometry), using lymphocytes from 21 healthy volunteers and 14 hemodialysis patients. The healthy volunteers, 11 women and 10 men, were ages 21–60 years (median 36 years). The hemodialysis patients, 6 women and 8 men, ages 26–75 years (median 60 years), were dialyzed two or three times a week for 4 h with dialysate containing magnesium at 0.50 mmol/L. As above, the study was performed according to the World Medical Association Declaration of Helsinki.

STUDY SETUP

Isolation of and treatment of lymphocytes. As described above in the main report, venous blood was obtained in Vacutainer Tubes containing heparin or polystyrene granules to obtain 4.5 mL of heparinized blood or 20 mL of defibrinated blood, respectively. The lymphocytes in the heparinized blood were used for SCDM determinations of iMg\textsuperscript{2+}{\textsubscript{L}}; the lymphocytes harvested from defibrinated blood were used for MCDM determinations of iMg\textsuperscript{2+}{\textsubscript{L}}.

Lymphocytes were isolated from whole blood as described by Huijgen et al. [8]. Some of the harvested cells were counted and differentiated, and the remaining cells were dissolved in buffer, as described above in Materials and Methods.

For determining iMg\textsuperscript{2+}{\textsubscript{L}} with a fluorescent probe, we loaded the cells with Mag-indo-1/AM by a modification of the method of Raju et al. [9]: Mag-indo-1/AM was added at a final concentration of 10.9 μmol/L to the cell suspension in buffer that also contained Pluronic and probenecid (concentrations as stated in Materials and Methods). This suspension was incubated for 30–60 min at 37 °C with gentle shaking. A portion of unloaded lymphocytes were reserved for measurement of autofluorescence by the MCDM.

After the loading, the lymphocytes were centrifuged at 150g for 12 min and the pellet was maintained at room temperature for another 30 min to allow the Mag-indo-1/AM to de-esterify. For cytosolic ion measurement, the cells were washed once and resuspended at 0.5 × 10^6 lymphocytes per milliliter in the same buffer but without fetal calf serum.

Using the SCDM, we could exclude dead cells from the final analysis by staining the cells with propidium iodide (Sigma; final concentration 12.5 μmol/L).

Measuring iMg\textsuperscript{2+} in lymphocytes. By SCDM, labeled lymphocytes were analyzed at 37 °C with a FACStarPLUS (Becton Dickinson Immuno Cytometry Systems) equipped with a 5-W watercooled argon-ion laser (Spectra Physics; Type 2020–05) and a 3-W watercooled UV laser (Spectra Physics; Type 2025–05). Mag-indo-1 was excited at 357 nm; the free Mag-indo-1 was detected with a DF405/20 BP filter and the bound probe with a DF485/22 BP filter. Electronic compensation was used to remove spectral overlap. The velocity of the fluid was such that 80–150 cells were counted per second; in total, 10 × 10^3 to 15 × 10^3 events per determination were recorded and analyzed. From the ratio (R) of the fluorescence intensities of the free and bound Mag-indo-1 in the living lymphocytes, we could calculate iMg\textsuperscript{2+}{\textsubscript{L}}:

\[
R = \frac{\text{fluorescence intensity of bound Mag-indo-1}}{\text{fluorescence intensity of free Mag-indo-1}}
\]

Forward scatter (FSC), sideway scatter (SSC), and the propidium iodide fluorescence were detected after excitation at 488 nm. The scatter intensities were detected with BP485/10 filters, and the fluorescence of propidium iodide was detected with a DF630/22 bandpass filter. In the FSC-SSC pattern, all lymphocytes were selected, whereas the fluorescence of propidium iodide allowed exclusion of the dead cells. Only the fluorescence of the living cells was used to calculate R.

By MCDM, labeled lymphocytes were measured at 37 °C with a dual-excitation and emission fluorometer (Amino Bowman Series 2 luminescence spectrometer). Excitation was at 347 nm; emission was monitored in 0.5-s intervals at 467 nm (free Mag-indo-1) and 428 nm (bound Mag-indo-1). To eliminate any fluorescence from probe leaking out of the cells, EGTA/EDTA (Sigma; final concentration 1.25 mmol/L) in Tris-HCl, 0.1 mol/L, was added to bind the extracellular iMg\textsuperscript{2+}. Because the autofluorescence detected never exceeded 1%, we calculated R directly from the fluorescence intensities (Eq. A1). Data were analyzed with the standard software on the SLM-Amino.

We used R, obtained from the fluorescence intensities determined by both methods, to calculate the iMg\textsuperscript{2+}{\textsubscript{L}} concentration as demonstrated before by Grynkiewicz et al. [10] in Eq. 1 for iMg\textsuperscript{2+}{\textsubscript{MBC}}, with R\textsubscript{min} as the ratio at
minimal iMg\(^{2+}\(_L\)) concentrations and R\(_{\text{max}}\) as the ratio at maximal iMg\(^{2+}\(_L\)) concentrations.

In determining K\(_d\), R\(_{\min}\) and R\(_{\text{max}}\), one must permeate the lymphocytes in such a way that Mg\(^{2+}\) can diffuse freely through the membrane, leaving the membrane more or less intact. The success of the permeation can be checked by using the SCDM: The shape and form of the cells can be studied with the FSC-SSC pattern, and the fluorescence intensity of Mag-indo-1 is studied only inside the cell. The following products for permeation have been tested: digitonin [29, 30], octylglucoside [31], saponin [32], FACS Lyse [31, 33], and 4Br-A23187 [7, 34]. When digitonin, octylglucoside, and saponin are used, the FSC-SSC pattern shifts towards the very small particles, indicating that the cell membrane is completely destroyed; with FACS Lyse and 4Br-A23187, the FSC-SSC pattern (and presumably the cell membrane) remains intact. However, the fluorescent properties of Mag-indo-1 change in the presence of FACS Lyse (which first permeates the cells and then fixates [31, 33]), whereas 4Br-A23187 does not alter these fluorescent properties. These results clearly demonstrate the usefulness of 4Br-A23187 as an ionophore for magnesium in lymphocytes, so we used 4Br-A23187 to permeate the cells for measurement of K\(_d\).

To obtain a minimal iMg\(^{2+}\(_L\)) concentration inside cells, for calculating R\(_{\min}\) and S\(_f\) (of free probe), we added the ionophore 4Br-A23187 (final concentration 8.0 \(\mu\)mol/L) and the chelators EGTA/EDTA (final concentration 1.25 mmol/L) in Tris-HCl, 0.1 mol/L, to the solution. The maximal iMg\(^{2+}\(_L\)) concentration for obtaining R\(_{\text{max}}\) and S\(_o\) (of free probe) was achieved by adding MgCl\(_2\) to this solution (final Mg\(^{2+}\) concentration 44.2 mmol/L).

To determine K\(_d\) by both the SCDM and the MCDM, a calibration curve was recorded by measuring R at several (usually 9) known iMg\(^{2+}\(_L\)) concentrations: After EGTA/EDTA and 4Br-A23187 were added, small amounts of 0.5 mol/L MgCl\(_2\) were added and R was measured. The free Mg\(^{2+}\) concentrations were calculated with the program CHELATOR [35]. We then performed linear regression over the (9) points for [(R - R\(_{\min}\))/(R\(_{\max}\) - R)] \(\times\) S\(_f\)/S\(_o\) against iMg\(^{2+}\(_L\)) and, as was shown in Eq. 1 for iMg\(^{2+}\(_L\)) determined K\(_d\) as the regression coefficient of the fit. The goodness of the fit is expressed by R\(^2\).

In general we reported the mean ± SE of a group. Statistical analyses were performed with the Mann–Whitney U-test and the statistical package SPSS (version 6.1.3) or the one-sample t-test when appropriate.

RESULTS AND DISCUSSION
The K\(_d\) of Mag-indo-1 with magnesium was 2.9 mmol/L (n = 5, R\(^2\) > 0.95) in the SCDM and 2.1 mmol/L (n = 12, R\(^2\) > 0.88) in the MCDM—different results but of the same magnitude, and in the same range as reported in the literature: 2.8 mmol/L [36] and 2.03 mmol/L [37]. For both methods, within-day and overall CVs were determined for samples from two different healthy persons (Table A1). The CV\(_{\text{within-day}}\) was slightly lower for the MCDM than the SCDM, whereas the CV\(_{\text{overall}}\) was slightly lower for the SCDM. Niemela et al. [18] reported a CV\(_{\text{overall}}\) of 11.0% for Mag-fura-2 in platelets, comparable with that for the SCDM. Others have found smaller values for CV\(_{\text{overall}}\) for a MCDM and Mag-fura-2: 6.5% in lymphocytes [7, 25], 3.6% in platelets [29].

**Effect of high extracellular iMg\(^{2+}\(_L\)) concentrations on iMg\(^{2+}\(_L\))**

We investigated the effect of increased extracellular iMg\(^{2+}\) on the intracellular iMg\(^{2+}\(_L\)) by using the SCDM. R (Eq. A1) was studied at a normal extracellular iMg\(^{2+}\) concentration, 0.42 mmol/L (R\(_{\text{normal}}\)), and a high concentration, 4.20 mmol/L (R\(_{\text{high}}\)). Under these conditions, the difference in R, defined as [(R\(_{\text{normal}}\) - R\(_{\text{high}}\))/R\(_{\text{normal}}\)] \(\times\) 100%, was respectively -4.4%, -1.3%, and -0.4% after incubation for 100, 150, and 200 min at this high concentration of extracellular magnesium. All these values fall within the error margin of R; thus, within 200 min or ~3.3 h, no effect of the high extracellular iMg\(^{2+}\) concentration was noted. Because both R\(_{\min}\) and R\(_{\max}\) were unaffected by the increase in extracellular iMg\(^{2+}\), even after a long exposure to such concentrations, R can be recorded instead of iMg\(^{2+}\(_L\)), obviating the time-consuming determinations of R\(_{\min}\) and R\(_{\max}\).

**Difference between methods for iMg\(^{2+}\(_L\)) in healthy volunteers.**

For both SCDM and MCDM, iMg\(^{2+}\(_L\)) was determined in blood collected at the same venipuncture from 21 healthy volunteers. After isolation, the cells were counted and the leukocytes were differentiated from the mixture of MBC in the samples. The mean lymphocyte concentration, relative to the total concentration of MBC, was 90% by the MCDM and 94% by the SCDM.

After staining and washing the cells, the fluorescent experiments were performed. In the MCDM, R decreased 12% after EGTA/EDTA was added to achieve a magnesium- and calcium-free extracellular buffer. This decrease demonstrates that fluorescent probe is present in the extracellular environment. In the SCDM, however, R was unaffected by the addition of the chelators EGTA/EDTA.

The mean iMg\(^{2+}\(_L\)) (±SE) in the healthy volunteers was 0.27 ± 0.01 mmol/L by the SCDM and 0.24 ± 0.01 mmol/L by the MCDM.

<table>
<thead>
<tr>
<th>MCDM</th>
<th>SCDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average iMg(^{2+}(_L))</td>
<td>0.34(^\circ)</td>
</tr>
<tr>
<td>CV(_{\text{within-day}}), %</td>
<td>4.7(^a)</td>
</tr>
<tr>
<td>Average iMg(^{2+}(_L))</td>
<td>0.37(^b)</td>
</tr>
<tr>
<td>CV(_{\text{within-day}}), %</td>
<td>18.3(^b)</td>
</tr>
<tr>
<td>Average CV(_{\text{within-day}}), %</td>
<td>5.4</td>
</tr>
<tr>
<td>Average CV(_{\text{overall}}), %</td>
<td>17.3</td>
</tr>
</tbody>
</table>

Number of experiments using the same one healthy volunteer: \(^a\) 4, \(^b\) 5, \(^c\) 9.
mmol/L by the MCDM (Table A2). The mean difference in iMg\textsuperscript{2+}\textsubscript{L} (iMg\textsuperscript{2+}\textsubscript{L} by SCDM – iMg\textsuperscript{2+}\textsubscript{L} by MCDM) was 0.03 ± 0.02 mmol/L.

Plotted the difference (SCDM – MCDM) vs the sum (SCDM + MCDM) of the iMg\textsuperscript{2+}\textsubscript{L} by both methods showed that the difference increased with increasing sum. The difference and the sum of the log(iMg\textsuperscript{2+}\textsubscript{L}) of both methods are shown in Fig. A1 [38]. The mean log difference was 0.06 (95% confidence interval, –0.01 to 0.13). Because the confidence interval includes 0, we conclude there is no bias between the two methods. Indeed, a one-sample t-test performed on the logarithmic difference in iMg\textsuperscript{2+}\textsubscript{L} gave P = 0.10 (not significant).

**Table A2. Mean ± SE iMg\textsuperscript{2+}\textsubscript{L} (mmol/L) of 21 healthy volunteers determined with SCDM and MCDM.**

<table>
<thead>
<tr>
<th>iMg\textsuperscript{2+}\textsubscript{L}</th>
<th>MCDM</th>
<th>SCDM</th>
<th>Difference (SCDM – MCDM)</th>
<th>Log difference [log(SCDM) – log(MCDM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.24</td>
<td>0.27</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>SE</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**iMg\textsuperscript{2+}\textsubscript{L} and serum Mg in healthy volunteers and hemodialysis patients.** We determined iMg\textsuperscript{2+}\textsubscript{L}, the distribution of iMg\textsuperscript{2+}\textsubscript{L} within a sample, and serum concentrations of Mg (iMg\textsuperscript{2+}\textsubscript{s} and tMg\textsubscript{s}) in both healthy volunteers and hemodialysis patients. The mean concentrations and the two-tailed probability by the Mann–Whitney test are summarized in Table A3.

Both iMg\textsuperscript{2+}\textsubscript{s} and tMg\textsubscript{s} were significantly greater in the patients (n = 14) than in the volunteers (n = 21), as others have also found [20, 39]. Kisters et al. [19] found no significantly increased tMg\textsubscript{s} or iMg\textsuperscript{2+}\textsubscript{s} in patients with renal insufficiency. In contrast, Markell et al. [39] found lower iMg\textsuperscript{2+}\textsubscript{s} in hemodialysis patients. This difference in serum magnesium concentrations is probably caused by differences in the magnesium concentration in the dialysate, 0.50 mmol/L (here and [19]) vs 0.375 mmol/L [39]. In the present study, the magnesium concentration in the dialysate (0.50 mmol/L) was lower than the concentration of iMg\textsuperscript{2+}\textsubscript{s} (0.68 mmol/L).

The mean iMg\textsuperscript{2+}\textsubscript{s} was slightly higher in the patients than in the volunteers (Table A3), but not significantly, probably because of the size of the two groups studied. Kisters et al. [19], also determining iMg\textsuperscript{2+}\textsubscript{s} in renal insufficiency patients and healthy volunteers, found the same results: The iMg\textsuperscript{2+}\textsubscript{s} was greater in the renal insufficiency group, but the difference was not significant.

The distribution in iMg\textsuperscript{2+}\textsubscript{L} (determined by using the distribution in R) for each hemodialysis patient ranged from 0.35 to 0.55 measuring units (1 unit is not equal to 1 mmol/L); for the healthy volunteers, the range was 0.32 to 0.56 measuring units.

Table A4 compares the mean iMg\textsuperscript{2+}\textsubscript{L} concentrations measured by the SCDM and the MCDM with the reported mean intracellular magnesium concentrations in lymphocytes as determined with MCDM and Mag-fura-2. The iMg\textsuperscript{2+}\textsubscript{L} concentration in the literature ranges from 0.15 to 0.57 mmol/L, with one extraordinary high concentration of 2.38 mmol/L reported [19]. The iMg\textsuperscript{2+}\textsubscript{L} concentrations determined in the present study are within the concentration range 0.15–0.57 mmol/L, and the %SD of the mean iMg\textsuperscript{2+}\textsubscript{L} concentration of healthy volunteers—22% (SCDM) and 25% (MCDM)—is within the range found in literature: 13–27%.

A possible explanation for the extremely high iMg\textsuperscript{2+}\textsubscript{L} found by Kisters et al. [19] is their use of digitonin to permeabilise the cells; all the other authors used 4Br-A23187. As already discussed, digitonin destroys the lymphocytes and thus the fluorescent probe is surrounded by buffer solution instead of the intracellular environment. The different R\textsubscript{min} and R\textsubscript{max} values obtained may explain such a high iMg\textsuperscript{2+}\textsubscript{L} concentration.

**Advantages of the methods.** iMg\textsuperscript{2+}\textsubscript{L} can be determined with a fluorescent probe by either flow cytometry (SCDM) or fluorometry (MCDM). The SCDM has some advantages over the MCDM: (a) The SCDM is not sensitive to extracellular probe leaking out of the cells into the surrounding buffer; (b) the distribution of iMg\textsuperscript{2+}\textsubscript{L} in a population of lymphocytes can be studied [28]; and (c) the SCDM can select a small population of cells, indicating that the purity of the sample is not important. On the other hand, the MCDM is easy to handle, relatively cheap, and available in almost every laboratory, whereas the SCDM required has to be equipped with an expensive UV laser. To determine whether the theoretical advantages of the SCDM could be mirrored in a practical situation, we used the flow cytometer and the fluorometer to study the iMg\textsuperscript{2+}\textsubscript{L} in healthy volunteers and hemodialysis patients.
The dissociation constant between the fluorescent probe and magnesium, \( K_d \), is needed for calculating the \( iMg^{2+} \), from the fluorescence intensity of the fluorescent probe. As demonstrated before [40], the \( K_d \) recorded in a buffer differs from that recorded in cells; the \( K_d \) also differs for each apparatus used. Therefore, the \( K_d \) had to be determined for each apparatus as well as inside the lymphocytes. For both the SCDM and the MCDM, the \( K_d \) has to be determined by disrupting the cell membrane in such a way that Mg\(^{2+}\) can diffuse freely through the membrane while the fluorescent probe remains inside the cell. In the SCDM, the disruption of the cells can be studied in the FSC-SSC pattern.

A. Effect of extracellular probe leaking out of the cells. Theoretically, the R that is measured after the addition of EGTA/EDTA is smaller than the true R: the fluorescence intensity of free Mag-indo-1 is overestimated because it is the sum of both extracellular and intracellular free probe, whereas the fluorescence intensity of bound Mag-indo-1 is the true fluorescence intensity, arising only from intracellular Mag-indo-1. Eq. A1 easily shows that the measured R is smaller than the true R. Because the other factors needed to calculate \( iMg^{2+} \) are unaffected, the \( iMg^{2+} \) now measured is smaller than the true \( iMg^{2+} \) (Eq. 1). Therefore, the \( iMg^{2+} \) measured with the MCDM is assumed to be less than the \( iMg^{2+} \) measured with the SCDM. This difference in \( iMg^{2+} \) increases as more fluorescent probe leaks out of the lymphocytes into the surrounding buffer.

When we determined the \( iMg^{2+} \) of 21 healthy volunteers with both the SCDM and the MCDM, the mean difference in \( iMg^{2+} \) (SCDM − MCDM) was 0.03 mmol/L and, as expected, the \( iMg^{2+} \) measured by the MCDM was lower than by the SCDM. Because the difference increases when the sum of \( iMg^{2+} \) by both methods increases, we calculated the logarithmic difference and the logarithmic sum. As discussed before, the 95% confidence interval for the logarithmic difference included 0, and a one-sample t-test demonstrated no bias between the two methods [38]. Therefore, the effect of the extracellular probe on the \( iMg^{2+} \) is negligible and the MCDM can be used without much difficulty.

B. Effect of the distribution of the magnesium concentration in a population of lymphocytes. Although the mean \( iMg^{2+} \) is the same for healthy volunteers and hemodialysis patients (Table A3), the distribution of \( iMg^{2+} \) within one sample might be different between these two groups. Examining the width of the distribution in the ratio R should give an indication about the width in distribution in \( iMg^{2+} \). However, the distribution of R was the same for both hemodialysis patients and healthy volunteers, 0.2 measuring units. Therefore, the distribution of \( iMg^{2+} \) as determined with the SCDM contains no extra information for this group of patients.

C. Effect of the purity of the cell population to be analyzed. Comparing the reproducibility of the SCDM and MCDM determinations of \( iMg^{2+} \) is a measure of the effects of the purity of the sample on \( iMg^{2+} \) and of the reproducibility of the isolation procedure on \( iMg^{2+} \). In the SCDM, \( iMg^{2+} \) is determined in lymphocytes only, whereas the MCDM determines \( iMg^{2+} \) in a mixture of MBC, in which the lymphocytes make up the majority of cells present (90%). Because the CVoverall for the SCDM and the MCDM are comparable, the different mixtures of cells have no effect on the precision of \( iMg^{2+} \).

In conclusion, the SCDM has the advantages of being insensitive to the interference of extracellular probe and to the purity of the sample and offers the possibility of studying the distribution of the intracellular ionized magnesium concentrations in a sample. The MCDM has the advantages of being easily accessible, available in most laboratories, and less expensive than the SCDM with a UV laser. In this study, however, we found no significant difference in the mean \( iMg^{2+} \) determined by both methods. Nor was a difference found in the width of the distribution of \( iMg^{2+} \) in hemodialysis patients and healthy volunteers. Moreover, the reproducibility in \( iMg^{2+} \) in both methods is similar, indicating that the purity of the sample as used here does not affect the reproducibility. Therefore, all of the theoretical advantages expected with use of the SCDM were of no effect in this practical situation. On the basis of these results and keeping financial considerations in mind, we conclude that the MCDM, the fluorometer, is the preferred method for determining \( iMg^{2+} \).
References


