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Plasma magnesium is inversely associated with Epstein-Barr virus load in peripheral blood and Burkitt lymphoma in Uganda

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ABSTRACT

Background: Epstein-Barr virus (EBV) causes endemic Burkitt lymphoma (eBL). EBV control was improved by magnesium (Mg^{2+}) supplementation in XMEN, an X-linked genetic disease associated with Mg^{2+} deficiency, high circulating EBV levels (viral loads), and EBV-related lymphomas. We, therefore, investigated the relationship between Mg^{2+} levels and EBV levels and eBL in Uganda.
Methods: Plasma Mg^{2+} was measured in 45 women with low or high circulating EBV levels, 40 pediatric eBL cases, and 79 healthy children. Mg^{2+} uptake by T-lymphocytes was evaluated in samples from healthy donors.
Results: Plasma Mg^{2+} deficiency (plasma level < 1.8 mg/dl) was more likely in women with high- vs. low-EBV levels (76.0% vs. 35%; odds ratio [OR] 11.3, 95% CI 2.14–60.2), controlling for age, and in eBL cases than controls (42.0% vs. 13.9%; OR 3.61, 95% CI 1.32–9.88), controlling for sex, age group, and malaria status. Mg^{2+} uptake by T-lymphocytes was related to extracellular Mg^{2+} concentration.
Interpretation: Plasma Mg^{2+} deficiency is associated with high EBV levels and eBL.

1. Introduction

Epstein-Barr virus (EBV) is causally related to about 197,000 cancer cases per year, including 100% of endemic Burkitt lymphoma (eBL); [1] 100% of anaplastic nasopharyngeal carcinoma; [2] 40% of Hodgkin lymphoma; [3] and 8% of gastric cancers [4]. However, there is no preventative vaccine, no method to control EBV spread, and no cost-effective way to identify high-risk groups for early cancer detection.

The discovery of the **X**-linked immunodeficiency with **m**agnesium (Mg^{++}) defect, high **E**BV viral load in blood, and high risk for EBV-related **n**eoplasia (XMEN) syndrome [5], suggests a novel role of Mg^{2+} in EBV control [5]. XMEN patients suffer loss-of-function mutations in the Mg^{2+} transporter 1 gene (*MAGT1*), which decreases extracellular uptake of free basal Mg^{2+} by natural killer (NK) and CD8+ T-lymphocytes [5]. Intracellular Mg^{2+} plays a second messenger role in regulating the expression of NKG2D receptors on NK and CD8+ T-lymphocytes, a critical step in the cytolytic response against EBV infection. Thus, the functional consequences of *MAGT1* abnormalities in XMEN are decreased intracellular free basal Mg^{2+} in NK and CD8+ T-lymphocytes; decreased expression of NKG2D receptors on the T cells; and impaired EBV control. These abnormalities lead to uncontrolled EBV infection, extraordinarily high circulating levels of EBV (viral loads), and increased risk of EBV-neoplasia [6]. Mg^{2+} supplementation in XMEN patients increases intracellular Mg^{2+} to the upper limit of the normal physiological range, restores NKG2D receptor expression on NK and CD8+ T-lymphocytes, normalizes EBV cellular-immune responses, and leads to effective suppression of EBV viral load [6]. To determine whether findings in XMEN may apply to the general population, we investigated the association between plasma Mg^{2+} levels and EBV viral load peripheral blood in healthy women and children with eBL in

Abbreviations: XMEN, the **X**-linked immunodeficiency with **m**agnesium (Mg^{2+}) defect, high **E**BV viral load in blood, and high risk for EBV-related **n**eoplasia; eBL, endemic Burkitt lymphoma; EBV, Epstein-Barr virus; Mg^{2+}, magnesium; *MAGT1*, magnesium transporter 1 gene; $MgCl_2$, magnesium chloride; CV, coefficient of variation

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2. Methods

Plasma Mg^{2+} levels were measured in a random sample of 45 Ugandan women with the sickle cell trait: 25 with high (upper-quartile) peripheral blood EBV viral load and 20 with low (lower-quartile) EBV viral load enrolled from the Sickle Cell Clinic at Mulago Hospital [7]. The women were selected from 233 women originally enrolled as mother-child pairs to study human herpesvirus 8 (HHV8) transmission in children with sickle cell disease in Uganda. EBV viral load in peripheral blood and saliva from the women was measured using quantitative polymerase chain reaction (qPCR) of DNA of the *EBNA1* gene [7]. We detected EBV in 72% of peripheral blood samples (median: 2.7 $\log_{10}$ copies/million white cells) and 79% of saliva samples (4.8 $\log_{10}$ copies/mL) [7]. Plasma Mg^{2+} was also measured in 40 children with newly diagnosed (pre-treatment) histologically proven eBL cases and 79 healthy controls from the same region as the cases enrolled in the Epidemiology of Burkitt Lymphoma in East African children and Minors (EMBLEM) study in Uganda [8].

Total plasma Mg^{2+} was measured using atomic emission spectroscopy with an iCAP6500 Duo Simultaneous ICP-OES instrument (Thermo-Fisher Scientific) [9]. Reliability of Mg^{2+} results was evaluated by testing two blinded samples from the women several months apart. Mg^{2+} levels in the children were measured once in duplicate samples. Mg^{2+} results from replicate tests were reproducible (r = 0.84, mean CV 3.5%), so the average results from all measurements are presented.

The Mulago Hospital Research and Ethics Committee, Uganda National Council for Science and Technology and the NCI Special Studies Institutional Review Boards gave ethical approval to conduct the study. Written informed consent was obtained from all participants.

Uptake of extracellular Mg^{2+} by T-lymphocytes isolated from healthy donors or Jurkat cells was evaluated by incubating the lymphocytes in media with different concentrations of magnesium chloride ($MgCl_2$): 0 mM −10 mM, and measuring the free (active) intracellular Mg^{2+} using the Mag-Indo 1 dye. The lymphocytes were incubated in assay buffer (AB: NaCl 120 mM, HEPES 20 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, and glucose 1.8 mg/mL, pH 7.4) for 20 min. The intracellular Mg^{+2} indicator Mag-Indo 1-AM (Invitrogen M1295) was loaded at 0.33 mM in the presence of Powerload (Invitrogen P10020) in AB in the dark for 20 min. The cells were washed and incubated in AB for 20 min, washed and re-suspended in AB. Flow cytometry was performed using a BD LSRII flow cytometer. Cells were acquired for 30 s (baseline) before adding media supplemented with different $MgCl_2$ concentrations and continuing acquisition for 5 min. All steps were done at room temperature. Kinetic flow cytometric analysis was done with FlowJo software package (FlowJo LLC), and results exported to Prism (GraphPad) for plotting. The experiment was repeated three times, and similar results were obtained.

Plasma Mg^{2+} levels in the women with high vs. low EBV viral load were compared using a nonparametric test with continuity correction. Because the sample size was larger in the children, the equality of mean Mg^{2+} levels in the children with vs. without eBL was evaluated using Student's *t*-test. Odds ratios [ORs] of association with Mg^{2+} deficiency, defined as values < 1.8 mg/dl (equivalent to < 0.7 mmol/L or 1.5 meq/L) [10], in the women or with BL in the children was estimated using logistic regression. ORs were adjusted for the age group in the women (< 35 vs. > =35 years) and for age group (0–4, 5–9, 10–16 years), sex, and malaria infection status in the children [8]. Because we hypothesize that EBV load is in the causal pathway for Mg^{2+} deficiency, we tested this possibility by adding EBV load to the adjusted model and checking whether the effect of Mg^{2+} deficiency on BL risk was attenuated. Statistical tests were two-sided.

3. Results

Table 1 shows characteristics of study subjects. Plasma Mg^{2+} levels in the women were negatively correlated with EBV viral load in peripheral blood (r = −0.30, p = 0.059). Plasma Mg^{2+} levels were

Table 1
showing characteristics of Ugandan subjects evaluated for relationship between plasma Mg^{2+} levels and peripheral blood EBV load (Women) or Burkitt lymphoma (Children).

	Women				Children				
Characteristic	Low EBV	High EBV	OR (95% CI)[a]	OR (95% CI)[b]	Controls[c]	BL[c]	OR (95% CI)[a]	OR (95% CI)[b]	OR (95% CI)[b]
sex									
Males	–	–	–	–	35 (44.3%)	27 (67.5%)	Ref.	Ref.	Ref.
Females	20	25	–	–	44 (55.7%)	13 (32.5%)	0.38 (0.17–0.85)	0.39 (0.15–0.98)	0.98 (0.90–5.10)
Age group, years									
0–4	–	–	–	–	28 (35.4%)	5(12.5%)	0.25 (0.08–0.76)	0.34 (0.09–1.28)	0.13 (0.01–1.05)
5–9	–	–	–	–	28 (35.4%)	20 (50.0%)	Ref.	Ref.	Ref.
10–16	–	–	–	–	23 (29.1%)	15 (37.5%)	0.91 (0.38–2.17)	1.10 (0.40–3.03)	0.34 (0.04–2.56)
17–34	15 (75.0%)	13 (52.0%)	Ref.	Ref.	–	–	–	–	–
35+	5 (25.0%)	12 (48.0%)	2.77 (0.77–9.97)	6.57 (1.61–37.1)	–	–	–	–	–
Malaria status									
Negative	–	–	–	–	31 (40.8%)	24 (63.2%)	Ref.	Ref.	Ref.
Positive	–	–	–	–	45 (59.2%)	14 (36.8%)	0.40 (0.18–0.90)	0.40 (0.16–1.01)	0.28 (0.06–1.25)
EBV load, $\log_{10}/10^6$ WBCs (SD)	–	–	–	–	6.37 (4.5)	12.6 (1.6)	3.13 (2.01–4.87)	–	2.87 (1.69–4.88)
Magnesium level									
> =1.8 mg/dl	13 (75.0%)	6 (24.0%)	Ref.	Ref.	11 (13.9%)	17 (42.5%)	Ref.	Ref.	Ref.
< 1.8 mg/dl	7 (25.0%)	19 (76.0%)	5.88 (1.60–21.5)	11.3 (2.14–60.2)	68 (86.1%)	23 (57.5%)	4.57 (1.87–11.2)	3.61 (1.32–9.88)	2.24 (0.40–12.7)

Notes: OR Odds ratio; 95% CI 95% Confidence Interval; BL Burkitt lymphoma; EBV Epstein Barr virus; SD standard deviation; WBC white blood cells.

[a] Crude ORs.

[b] Adjusted for one another.

[c] Totals do not sum to 40 BL cases and 79 controls for some categories because of missing data.

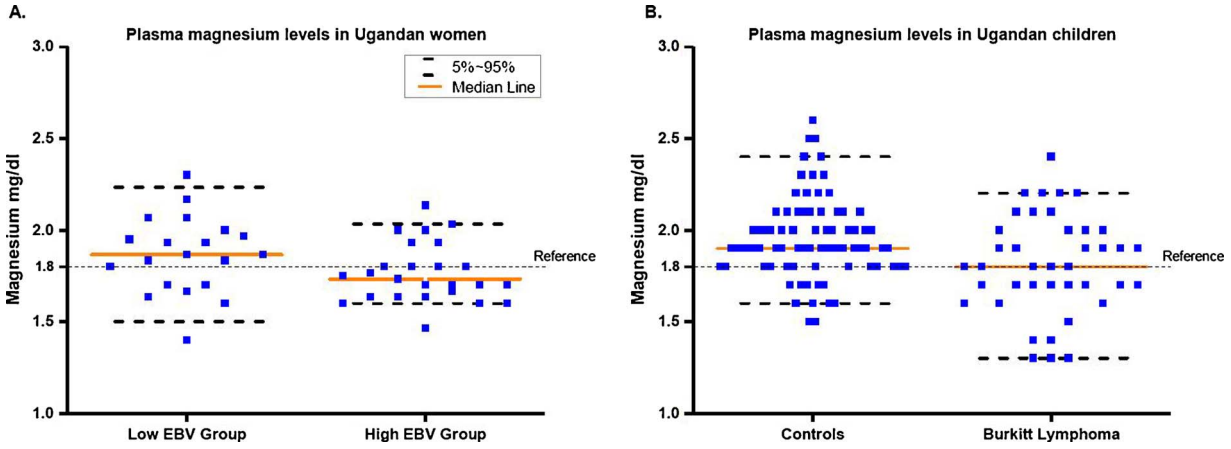

Fig. 1. Plasma magnesium levels in women with high or low EBV levels in blood and children with or without BL: Panel A: Dot plot showing average plasma Mg^{2+} levels (mg/dl) in women with low (lower-quartile) vs. high (upper-quartile) EBV viral load. Each dot is a single subject. Panel B: Dot plot showing plasma Mg^{2+} levels (mg/dl) in children with and without BL in Uganda. Each dot represents a single subject. A reference line for Mg^{2+} deficiency (< 1.8 mg/dl) is included in both panels. Lines show the 5%, 50% and 95% percentiles.

significantly decreased (p = 0.014) in women with high in comparison to women with low EBV viral load in peripheral blood (1.73 vs. 1.87 mg/dl, Fig. 1A). In crude analysis, the Mg^{2+} deficiency was more likely in women with high EBV vs. low peripheral blood EBV viral load (76.0% vs. 35.0%; OR = 5.88, 95% CI 1.60–21.5; Table 1). The results were more pronounced when we controlled for age group (OR = 11.3, 95% CI 2.14–60.2). In contrast, the correlation between plasma Mg^{2+} levels and EBV in saliva was less pronounced (r = −0.018, p = 0.08), and Mg^{2+} levels were not different in those with high vs. low EBV levels in saliva (p = 0.214).

Plasma Mg^{2+} levels in the children with eBL were significantly decreased in comparison with those in the healthy children (p = 0.0016), and the mean plasma Mg^{2+} level for eBL patients was consistent with mild Mg^{2+} deficiency (1.81 versus 1.95 mg/dl, Fig. 1B). Interestingly, plasma Mg^{2+} levels in the BL cases did not differ by tumor anatomic site: the face only (1.8 mg/dl), face and abdomen (1.83 mg/dl), abdomen only (1.82 mg/dl). Levels were significantly depressed in the three children with involvement of the central nervous system and other sites (1.47 mg/dl). Mg^{2+} deficiency was more likely in eBL cases than in the healthy children (42.5% vs. 13.9%; OR = 4.57, 95% CI 1.87–11.2). The relationship between Mg^{2+} deficiency and eBL remained when adjusting for age group, sex, and malaria positivity (OR = 3.61, 95% CI 1.32–9.88), but it was attenuated and lost significance when we included EBV load in the model (OR = 2.24, 95% CI 0.40–12.7; Table 1). We note that life-threatening levels of Mg^{2+} deficiency, defined as levels < 1.2 mg/dl [10], were not observed in the study.

Concentration-dependent uptake of extracellular Mg^{2+} was observed in T lymphocytes from healthy donors incubated in media supplemented with $MgCl_2 \geq 0.3$ mM, but not in lymphocytes incubated in media with $MgCl_2 < 0.3$ mM (Fig. 2). The slope of the curve, which indicates the rate of Mg^{2+} uptake, was directly proportional to the concentration of $MgCl_2 \geq 0.62$ mM. We obtained similar results using Jurkat cells (data not shown).

4. Discussion

The discovery in XMEN patients that a defect in intracellular free basal Mg^{2+} in humans can severely compromise EBV cellular immunity [5], and that the defect could be reversed by raising extracellular Mg^{2+} levels [6] prompted us to hypothesize that Mg^{2+} deficiency may be relevant for uncontrolled EBV infection in Africa. Our findings of strong, consistent, and independent inverse associations between plasma Mg^{2+} levels and peripheral blood EBV viral load in the women, and with eBL risk in the children are consistent with our hypothesis. Overall, we found evidence of mild Mg^{2+} deficiency in about 14% of the healthy children, about 35% of women with low EBV viral load, and about 43% of the children with eBL. These results suggest that mild Mg^{2+} deficiency is relatively frequent and may be relevant for EBV control in Uganda.

The absence of systematic studies of Mg^{2+} levels in general populations in African countries, including Uganda, precludes detailed discussion of our results. Nonetheless, mild Mg^{2+} deficiency has been reported in some African countries, including in 15% of 160 pregnant normotensive women attending a clinic in Ghana [11] and 68% of 101 patients admitted for emergency intra-abdominal surgery in Ghana [12]. These results suggest that mild Mg^{2+} deficiency is not uncommon in Africa, particularly in countries with high eBL incidence [13]. Our results should encourage investigators interested in conducting hypothesis-driven research to characterize the distribution Mg^{2+} deficiency in African populations and to determine its causes. Possible causes include consumption below the recommended dietary intake, which has been shown to occur frequently even in developed countries, including the US and UK [14], and is likely to be more so in African populations relying on whole grains, roots, and tubers for their Mg^{2+} intake [15]. If the African diets fall short of the recommended daily allowance, depression of plasma Mg^{2+} levels may lead to a concentration-dependent reduction in Mg^{2+} uptake by NK and CD8+ T-lymphocytes, as suggested by the results from our in vitro experiments, and, consequently, impairment in EBV controls as observed in XMEN patients [6]. We acknowledge that our findings are based on small numbers and that our interpretations are somewhat speculative, but they are consistent with the implied underlying mechanism based on findings in XMEN patients. These results should encourage the design of new studies to confirm or refute the hypothesis that mild Mg^{2+} deficiency is a risk factor for poor EBV control of BL in Africa.

Authorship contributions

SMM, KB, and MJL conceived the idea and designed the study; SMM supervised the field work and coordinated sample testing and performed analysis; EAE, JJG, contributed to analyses and interpretation of data, SJR, IO, HN, IDL, MDO, and CMN performed fieldwork; DW, VM, and JR performed laboratory tests; SMM and JR drafted the manuscript; all authors reviewed and approved the final manuscript.

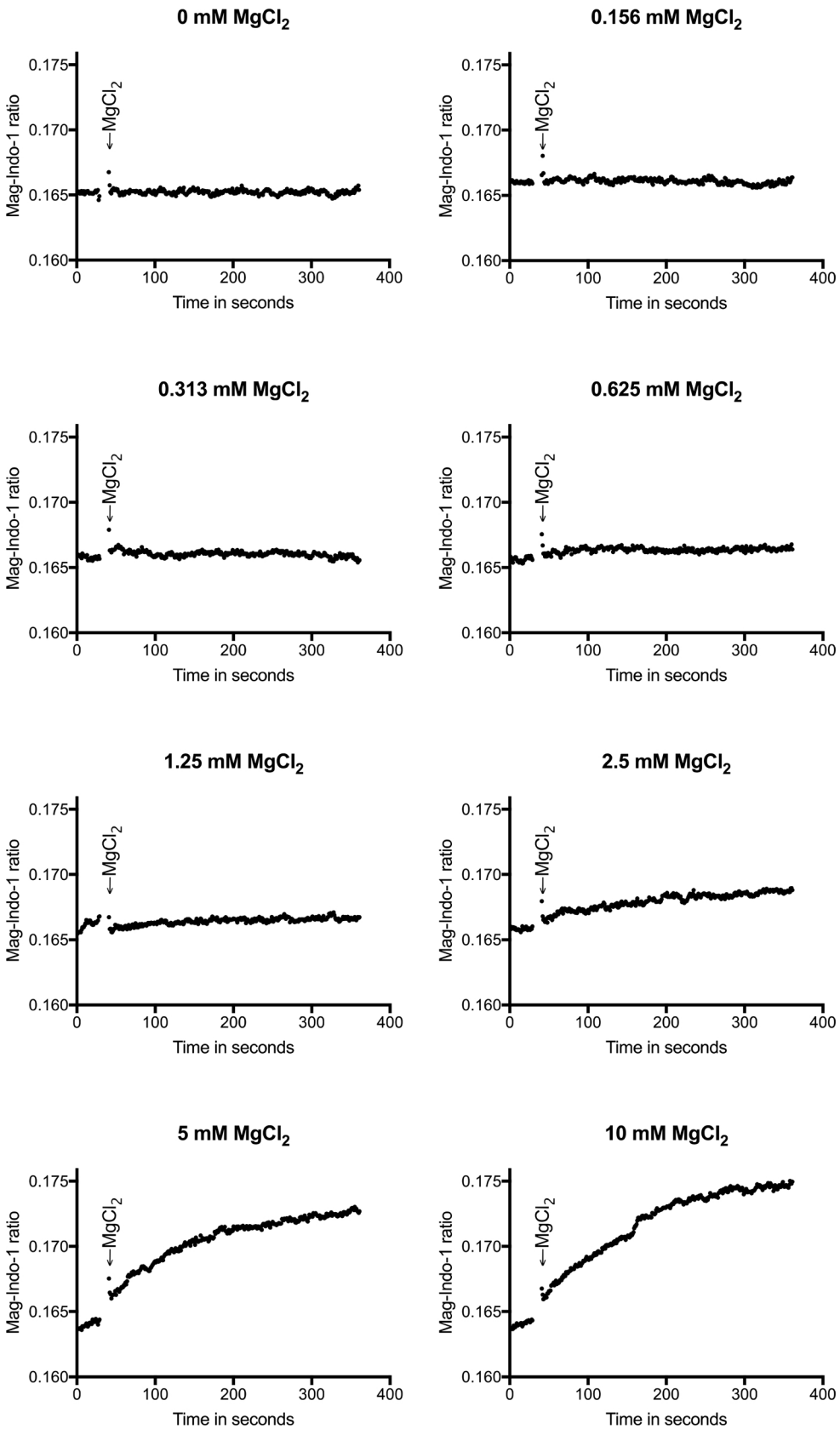

Fig. 2. Extracellular Mg^{2+} concentration influences the free intracellular Mg^{2+} content in T lymphocytes. Intracellular magnesium concentration in response to the addition of extracellular magnesium chloride ($MgCl_2$) was measured by flow cytometry using the fluorescent radiometric magnesium indicator Mag-Indo1 in T cells from healthy controls. Arrows correspond to the time of addition of $MgCl_2$. Extracellular $MgCl_2$ concentration for each condition is shown above each graph.

Disclosure of conflicts of interest

None declared.

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