Renal anaemia and EPO hyporesponsiveness associated with vitamin D deficiency: the potential role of inflammation

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ABSTRACT

Resistance to erythropoiesis-stimulating agents (ESAs) has been observed in a considerable proportion of patients with chronic kidney disease (CKD) and it is reportedly associated with adverse outcomes, such as increased cardiovascular morbidity, faster progression to end-stage renal disease (ESRD) and all-cause mortality. The major causes of ESA resistance include chronic inflammation producing suppressive cytokines of early erythroid progenitor proliferation. In addition, pro-inflammatory cytokines stimulate hepcidin synthesis thus reducing iron availability for late erythropoiesis. Recent studies showing an association in deficiencies of the vitamin D axis with low haemoglobin (Hb) levels and ESA resistance suggest a new pathophysiological co-factor of renal anaemia. The administration of either native or active vitamin D has been associated with an improvement of anaemia and reduction in ESA requirements. Notably, these effects are not related to parathyroid hormone (PTH) values and seem to be independent on PTH suppression. Another possible explanation may be that calcitriol directly stimulates erythroid progenitors; however, this proliferative effect by extra-renal activation of 1α-hydroxylase enzyme is only a hypothesis. The majority of studies concerning vitamin D deficiency or supplementation, and degree of renal anaemia, point out the prevalent role of inflammation in the mechanism underlying these associations. Immune cells express the vitamin D receptor (VDR) which in turn is involved in the modulation of innate and adaptive immunity. VDR activation inhibits the expression of inflammatory cytokines in stromal and accessory cells and up-regulates the lymphocytic release of interleukin-10 (IL-10) exerting both anti-inflammatory activity and proliferative effects on erythroid progenitors. In CKD patients, vitamin D deficiency may stimulate immune cells within the bone marrow micro-environment to produce cytokines, inducing impaired erythropoiesis. Immune activation involves the reticuloendothelial system, increasing hepcidin synthesis and functional iron deficiency. Consequences of this inflammatory cascade are erythropoietin (EPO) resistance and anaemia. Given the key role of inflammation in the response to EPO, the therapeutic use of agents with anti-cytokines properties, such as vitamin D and paricalcitol, may provide benefit in the prevention/treatment of ESA hyporesponsiveness.

INTRODUCTION

Although the pathogenesis of anaemia of CKD patients is multifactorial, it mainly results from the erythropoietic hypoproliferative state in turn due to relative insufficiency in the erythropoietin (EPO) production of failing kidneys [1]. Since the late 1980s, the use of ESAs has revolutionized the management of renal anaemia, significantly improving patient quality of life and avoiding the need for blood transfusion. Nevertheless, ∼5–10% [2] of patients exhibits an inadequate response to ESAs. In this setting, the definition of EPO hyporesponsiveness/resistance has been introduced to identify the inability to

achieve or maintain target haemoglobin (Hb) levels despite higher than usual doses of ESAs [3]. Several studies demonstrate an association between EPO resistance and poor clinical outcomes, with increased cardiovascular morbidity, faster progression to end-stage renal disease and all-cause mortality [4–7]. Higher ESA requirements may lead to an increased risk for adverse outcomes due to the underlying factors affecting EPO response, such as inflammation, and the potential non-erythropoietic effects of greater administered ESA doses. Identification of causes that enhance EPO responsiveness can optimize the management of anaemia management improving both the financial costs and safety of ESA therapy [8]. However, the pathway inducing inflammation-mediated EPO resistance has yet to be determined.

In vivo studies suggest that deficiencies in the vitamin D axis may be an additional pathophysiological co-factor of specific anaemia subtypes, such as renal anaemia and anaemia due to inflammation [9, 10]. In addition, data are available showing an inverse association between vitamin D levels and EPO requirements in CKD patients [11]. The wide array of biological actions exerted by vitamin D and its analogues includes modulation of the immune system mediated through anti-inflammatory effects. Native vitamin D supplementation and paricalcitol therapy have been associated with an improvement in biomarkers of inflammation [12–14].

The aim of this review is to assess the role of inflammation in the pathogenesis of renal anaemia and EPO resistance associated with vitamin D deficiency and potential therapeutic implications in the future.

CONTROL OF ERYTHROPOIESIS AND RENAL ANAEMIA

The mature red blood cell (RBC) is the final product of a complex series of events in the bone marrow known as erythropoiesis. Under normal conditions, this process results in a cell production rate that ensures a constant red cell mass. Erythropoiesis is initiated at the time a pluripotent stem cell commits to erythron and depends on properties of progenitor and precursor erythroid cells and their interactions with the haemopoietic micro-environment. Stem and erythroid cells are in intimate contact with stromal cells (fibroblasts, adipocytes, macrophages and endothelial cells), accessory cells (monocytes, T-lymphocytes) and the extracellular matrix. Within this micro-environment, the erythron cascade is regulated by growth factors/cytokines produced by stromal and accessory cells or elsewhere (i.e. erythropoietin). In the first stages of erythropoiesis (erythroid differentiation and erythropoietin-independent phase), many types of cytokines have stimulatory and augmenting effects on haematopoietic and early erythroid progenitors (BFU-e) [15, 16]. Under pathological conditions, such as chronic disease anaemia and inflammation, suppressive cytokines derived from accessory cells [interleukin-6 (IL-6), interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α)], negatively influence differentiation and proliferation activities [17]. In the late phase of erythropoiesis, erythropoietin is the essential stimulus to erythroid maturation and the lack of this hormone is the major cause of CKD anaemia [1]. In addition to EPO, iron availability for erythroid precursors is needed. Hepcidin, a small polypeptide produced and secreted by the liver, is a key mediator of systemic iron homoeostasis-regulating absorption and utilization. In CKD patients, multiple forms of interference on iron metabolism as well as inhibition of iron release from the reticuloendothelial system occur. Excessive hepcidin production by pro-inflammatory cytokines encoding gene expression contributes to the functional iron deficiency and associated renal anaemia and EPO resistance [18–20].

CKD-MBD, ANAEMIA AND EPO RESISTANCE

The hyperparathyroid state induces anaemia in patients with normal kidney function [21]. In secondary hyperparathyroidism of CKD patients, the high levels of circulating PTH have multiple biological effects, including an unfavourable influence on the anaemia of CKD patients [22]. Possible pathogenic mechanisms of this relationship may be PTH-direct effects on inhibition of early erythroid progenitors, endogenous EPO synthesis and RBC survival and loss [23–25]. On the other side, the most acknowledged, indirect, negative effect of PTH on bone marrow cellularity is through the induction of fibrosis [26]. In HD patients, the surgical and pharmacological suppression of PTH release an improvement in anaemia [27, 28]. Hyperphosphataemia and the increase in serum alkaline phosphatase are also associated with CKD anaemia and EPO hyporesponsiveness [29, 30]. Recent studies showing the association among vitamin D deficiency, low Hb levels and EPO resistance suggest a new pathophysiological co-factor of CKD anaemia (Table 1).

VITAMIN D DEFICIENCY IN CKD ANAEMIC PATIENTS

Vitamin D is synthesized in the skin or derived from nutritional sources and is transported to the liver, where it is metabolized to 25-hydroxivitamin D [25(OH)D$_3$], which is the main circulating form of vitamin D. The second hydroxylation takes place mainly in the kidney, where the 1α-hydroxylase enzyme converts 25(OH)D$_3$ to the active hormone 1,25-dihydroxyvitamin D [1,25(OH)$_2$D$_3$], the hormone that acts on mineral and bone metabolism. In the course of CKD, vitamin D abnormalities not only include the progressive loss of kidney function to form 1,25(OH)$_2$D$_3$, but also the capacity to maintain serum 25(OH)D$_3$ levels. This latter deficiency is due to insufficient sunlight exposure in chronically ill patients, malnutrition with dietary exclusion of vitamin D-rich food, urine loss in proteinuric nephropathies and effluent loss in peritoneal dialysis [8, 31]. In addition, with the progressive reduction in GFR renal megalin expression decreases and in turn reduces the renal uptake of 25 (OH)D$_3$ [32].

Previous studies have shown that vitamin D has pleiotropic effects which affect many organ systems, including the haemopoietic system. 1,25(OH)$_2$D$_3$ exerts its action by binding to the

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Table 1. Studies reporting the association between anaemia or EPO resistance and vitamin D deficiency in CKD patients

Authors	Patients	Form of vitamin D deficiency	Anaemia	EPO resistance
Kendrick [35]	16 301 CKD not requiring dialysis (NHANES III)	$25(OH)D_3$	•	
Patel NM (2010) [9]	1661 early CKD (SEEK)	$25(OH)D_3$,	•	
		$1,25(OH)_2D_3$	•	
Perlstein TS (2011) [10]	139 CKD >60-year-old	$25(OH)D_3$	•	
Kiss Z (2011) [11]	142 HD	$25(OH)D_3$		•
Sola et al. (2011) [45]	140 CKD stage 3–5 not on dialysis	$25(OH)D_3$	•	

vitamin D receptor (VDR), a member of a nuclear receptor superfamily present in several tissues, such as bone marrow, stromal and accessory cells [33]. In blood samples drawn for DNA analysis, VDR genotype was found to be linked to Hb values and EPO dose requirements in dialysis patients [34]. More recent analysis of the Third National Health and Nutrition Examination Survey (NHANES III) has provided evidence of a linear association between $25(OH)D_3$ concentration and the degree of anaemia in CKD patients not requiring dialysis [35]. An inverse association between $25(OH)D_3$ levels and EPO resistance index was shown in HD patients [11]. In a large cohort of early CKD subjects, $25(OH)D_3$ and $1.25(OH)_2D_3$ deficiency were independently associated with decreased Hb values and anaemia. Finally, patients with severe deficiency of both native and active vitamin D have a greater prevalence of anaemia than subjects with low serum levels of a single form of vitamin D [9]. It is intriguing that lower $1.25(OH)_2D_3$ levels were associated with higher hepcidin, thus suggesting a link between vitamin D deficiency and anaemia in CKD patients [36].

The administration of vitamin D and analogues has been associated with an improvement of anaemia and/or a reduction in EPO requirements. High-dose oral alfacalcidol showed a positive impact on anaemia in a small group of HD patients with a good iron status and efficacious dialysis parameters [37]. Goicoechea et al. (1998) demonstrated that i.v. calcitriol improved Hb levels and reduced the need for EPO in HD patients [38]. A significant increase in Hb and haematocrit was obtained after 4 months of treatment with calcitriol in a group of CKD patients undergoing HD and on conservative management. In this study, active vitamin D administration increased BFU-e proliferation in vitro and ex vivo [39]. Indeed, no randomized clinical trial (RCT) studies on treatment with vitamin D, calcitriol or analogues have examined the Hb end point in CKD [40]. Responders to calcitriol therapy showed a maintenance in EPO dose, which was in contrast with non-responders [23]. Recently, cholecalciferol supplementation allowed a reduction in the dose of darbepoetin (DPO) in HD patients and the same result for the requirement of EPO was observed with ergocalciferol, with no impact on markers of mineral metabolism [41].

The positive effects of vitamin D on both anaemia and EPO requirements, during CKD, could be related to its suppressive effects on PTH [23, 42]. Studies on the association between the decrease in PTH following the use of calciomimetics and the reduction in DPO and EPO doses strengthen this hypothesis [43, 44]. Indeed, the associations among vitamin D deficiency with anaemia, EPO responsiveness and vitamin D supplementations with an improvement of the Hb level and EPO need are not related to PTH values, and seem to be independent of PTH suppression [9, 39, 45]. Another possible explanation is that active vitamin D directly stimulates erythroid progenitors. VDRs have been discovered in several non-renal tissues, including the bone marrow [46]. Furthermore, many tissues have the 1α-hydroxylase enzyme and are then able to locally activate $25(OH)D_3$. Normalization of native vitamin D levels may provide an adequate substrate for local production of $1,25(OH)_2D_3$ in the bone marrow via extra-renal activity of 1α-hydroxylase enzyme [47]. It has been reported that haematons (the buffy coat of the bone marrow including erythroid precursors and stromal cells) contain significantly higher concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ than bone marrow plasma [39]. However, the direct activation of erythroid progenitors proliferative activity by local $1,25(OH)_2D_3$ is only one hypothesis. The majority of studies regarding vitamin D deficiency or supplementation and the extent of (renal) anaemia suggest a central role of inflammation in the mechanisms underlying these associations [10, 35, 47–49].

CKD ANAEMIA OF INFLAMMATION

Similar to atherosclerosis and many chronic disease states, CKD is largely considered as an inflammatory condition. Interestingly, the inflammatory state is an important cause of post-transplant anaemia, probably by inducing erythropoietin resistance [50]. In the past and more recently, evidence has accumulated for the association between inflammation, anaemia and EPO hyporesponsiveness in CKD patients. Several studies have demonstrated that C-reactive protein (CRP) levels positively correlate with the severity of the anaemia and EPO dose in patients on HD and peritoneal dialysis [51–54]. Serum

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levels of other inflammatory cytokines, such as IL-6, closely reflect CRP concentrations. Low Hb levels were accompanied by higher levels of both inflammatory markers (IL-6 and CRP) in HD patients and IL-6 was found to antagonize the response to EPO [55, 56]. It was observed that in HD patients with EPO resistance there were higher levels of ferritin, CRP and IL-6 compared with controls without anaemia and functional iron deficiency [57, 58]. Malyszko *et al.* (2009) extended this relationship to TNF-α, hepcidin and pro-hepcidin [59].

Cytokines may affect erythropoiesis at different stages. Immune activation involves accessory cells in the haemopoietic micro-environment and T-lymphocytes produce TNF-α and IFN-γ and monocytes TNF-α and IL-6, respectively. These pro-inflammatory cytokines inhibit erythroid progenitor cell proliferation and antagonize the anti-apoptopic action of EPO [60, 61]. It is thought that this direct negative effect on erythroid progenitors is primarily due to alterations in the sensitivity to EPO. TNF-α and IL-1 affect EPO synthesis *in vitro* and cause a dose-dependent inhibition of hypoxia-induced EPO production in the Hep3b cell line [62]. Inflammation also limits the available iron for erythropoiesis and, in CKD patients, EPO hyporesponsiveness often can be explained by functional iron deficiency. Increased levels of circulating cytokines (IFN-γ, TNF-α, IL-1 and IL-6) induce macrophages of the reticuloendothelial system to more readily take up and retain the iron [17]. In anaemia due to inflammation, hepatic hepcidin synthesis increases as a consequence of IL-6 induction, mediated through bone morphogenetic protein (BMP) signalling [19, 63]. Raised hepcidin levels cause iron retention in macrophages and enterocytes with a consequent reduction in the availability of iron for erythropoiesis which leads to impaired haeme synthesis [64–66]. In the bone marrow of patients with CKD, anaemia of inflammation stromal and accessory cells makes up a substantial proportion of the haemopoietic micro-environment (Figure 1).

Okonko *et al.* [67] obtained *in vitro* the abrogation of the BFU-e suppressive effects of uraemic serum in the presence of TNF-α-neutralizing antibodies. In EPO-resistant patients on HD, oral administration of pentoxifylline for 4 months improved Hb levels and this result was accompanied by a decrease in T-cell generation of TNF-a and IFN-γ [68]. More recently, Ferrari *et al.* [69] found that pentoxifylline improves CKD anaemia and reduces circulating IL-6. These results were associated with increased TSAT, suggesting improved iron mobilization through a pentoxifylline-mediated modulation of hepcidin. IL-6 inhibition and CRP lowering was associated with an increase in Hb levels in patients with rheumatoid arthritis treated with tocilizumab (anti-IL-6 receptor antibody) [70]. In Castleman disease, tocilizumab-down-regulating hepcidin synthesis improved the anaemia of inflammation [71]. In dyslipidaemic and anaemic patients undergoing HD, treatment with 80 mg fluvastatin for 8 weeks resulted in a decrease in CRP and hepcidin levels [72]. In a parallel study, low doses of simvastatin were associated with a tendency to increase the response to exogenous EPO [73]. Dialysis modalities and materials have been linked with the degree of inflammation, anaemia and EPO requirements. Movilli *et al.* [74] found a decrease in CRP levels and EPO dose after 6 months of switching
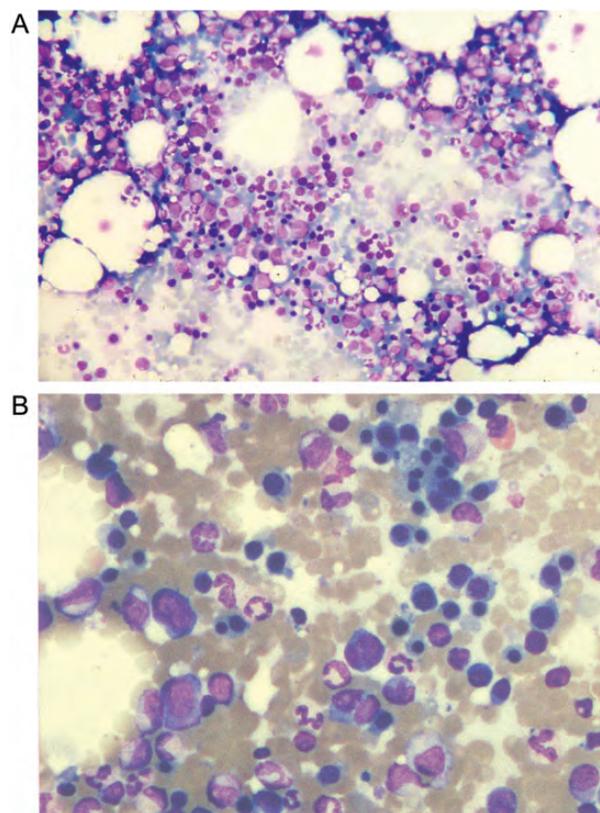

FIGURE 1: Histopathological morphology of bone marrow in (**A**) CKD (stage 5D) patient with increased serum markers of inflammation, anaemia and EPO resistance: a large number of stromal cells (adipocytes) instead of haemopoietic cells and in (**B**) control subject with normal erythropoietic cascade. (*Icardi A, private archive*).

from low-flux HD to post-dilutional on-line HDF. Recently, the positive effects of a vitamin E-coated polysulfone membrane on serum levels of inflammatory markers (CRP, IL-6) and EPO resistance has been reported [75].

ANTI-INFLAMMATORY EFFECTS OF VITAMIN D AND ANALOGUES: A THERAPEUTIC OPTION FOR CKD-RESISTANT ANAEMIA?

The role of vitamin D as a regulator of the immune system is well-established. Immune cells are targets for active vitamin D, modulating innate and adaptive immunity. VDR activation inhibits the expression of inflammatory cytokines, including IL-1, IL-6, TNF-α and IFN-γ, in accessory cells and in the serum [12, 33, 76]. VDR up-regulates the release of IL-10 (a cytokine exerting both anti-inflammatory activity and positive effects on erythroid proliferation) from lymphocytes [12, 33]. Aucella *et al.* [39] suggested that the improvement of erythropoiesis induced by calcitriol in CKD patients could be related to its suppressive effects on inflammatory cytokines. In addition, anti-IL-6 expression by activated VDRs may down-regulate the BMP-responsive element signalling pathway with a consequent decrease in hepatic hepcidin overproduction. The

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inhibition of the vitamin D analogue paricalcitol on renal inflammation was demonstrated in a mouse model of obstructive nephropathy: the reduced infiltration of T-lymphocytes and macrophages in the obstructed kidney was accompanied by a decrease in TNF-α mRNA expression in a dosage-dependent manner [77]. The *in vitro* inhibition of TNF-α release by paricalcitol was confirmed in cultures of human peripheral blood mononuclear cells in the presence or absence of high-inflammatory lipopolysaccharide (LPS) [78]. More recently, Guerrero *et al.* [79] showed that the increase in LPS-induced plasma TNF-α levels in uraemic rats was significantly reduced by the administration of either calcitriol or paricalcitol. This decrease was more accentuated after treatment with paricalcitol than with calcitriol. Paricalcitol administration at the start of the HD session may attenuate the inflammatory cytokine induction and expression during treatment, according to a preliminary report [80]. In the first randomized, placebo controlled trial examining the effects of oral paricalcitol on albuminuria and inflammation in CKD patients not requiring dialysis, the authors described a dose-dependent decline of the CRP plasma level in the paricalcitol group, whereas the placebo group was observed to have a 1.5-fold increase in baseline CRP. After withdrawing the analogue administration, CRP values showed a rebound effect, not reaching statistical significance [13]. In a small group of HD patients with 25(OH)D$_3$ insufficiency, cholecalciferol supplementation and paricalcitol therapy increased VDR expression in monocytes and reduced inflammatory cytokine plasma levels (IL-8, IL-6, TNF-α) [14]. In a large population of individuals with native vitamin D deficiency (65% with CKD), a greater prevalence of anaemia was found with a higher level of ferritin and a lower level of TIBC compared with those having normal 25(OH)D$_3$. This association suggested a condition of chronic inflammation leading to an ineffective late phase of erythropoiesis in vitamin D-deficient patients [47]. Daily supplementation of cholecalciferol in patients with congestive heart failure was able to increase IL-10 serum levels and avoid an up-regulation of TNF-α [81]. Recently, Bucharles *et al.* [82] observed a significant reduction in CRP and IL-6 levels due to cholecalciferol treatment in 30 HD patients without hyperparathyroidism, possibly suggesting an improvement of cardiac dysfunction.

In contrast, no association of vitamin D deficiency with inflammation was reported in other studies [83–85]. Moreover, supplementations with either native vitamin D or with active analogues showed no significant effects on inflammatory marker change [86, 87]. Taken together these data could raise concerns over whether vitamin D supplementation, either native or active, can be considered an actual strategy for correction of CKD anaemia of inflammation.

Last, no RCTs are available that have evaluated different effects of native vitamin D, calcitriol or analogues on haemoglobin as main outcome measure in CKD [40].

The association of vitamin D levels and inflammation status and anaemia grade in CKD patients was extensively examined in a cross-sectional study regarding 16 301 participants in the NHANES III. Within the whole population, age-adjusted and graded decreases in 25(OH)D$_3$ levels and Hb concentrations relating to an increase in CRP levels across
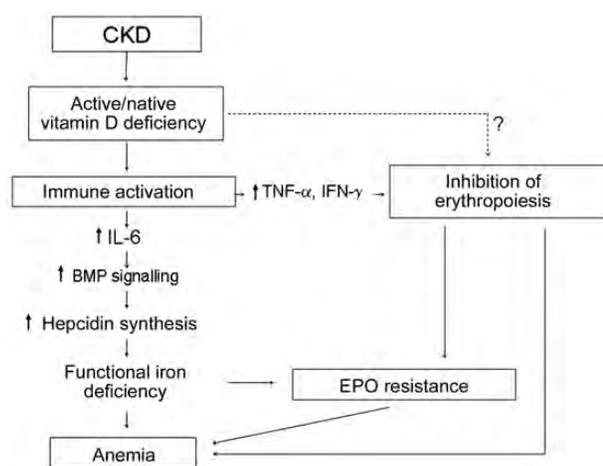

FIGURE 2: Effects of Vitamin D deficiency on inflammation and EPO-resistant anaemia.

detrimental estimated GFR categories were observed. This study showed an independent association of decreased 25(OH)D$_3$ concentrations and increased CRP levels with anaemia, thus suggesting that 25(OH)D$_3$ deficiency could be related to lower Hb values also via an inflammatory mechanism [35]. In CKD patients, native and active vitamin D deficiencies stimulate immune cells of the bone marrow micro-environment to produce pro-inflammatory cytokines with inhibition of erythropoiesis. In addition, immune activation involves the reticuloendothelial system, enhancing hepcidin synthesis with a decline of iron availability. All these inflammatory mechanisms lead to EPO resistance and anaemia (Figure 2).

Given the role of inflammation in the CKD anaemia, agents with anti-inflammatory properties, such as vitamin D, might be beneficial in the treatment of EPO-resistant patients. In a prospective study on 158 HD subjects, concerning the effects of cholecalciferol supplementation on mineral metabolism, inflammation and cardiac dimension parameters, the reduction in vitamin D deficiency allowed significant declines of CRP levels and DPO dose [88]. Future interventional studies are needed to confirm these hypotheses and to identify vitamin D and analogues as preventive agents and/or therapeutic drugs in the cure of CKD anaemia of inflammation and EPO hyporesponsiveness.

CONFLICT OF INTEREST STATEMENT

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