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Oxidative Stress Due to Aluminum Exposure Induces Eryptosis Which Is Prevented by Erythropoietin

Daiana M. Vota,[1,2] Renée L. Crisp,[1,3] Alcira B. Nesse,[1,2] and Daniela C. Vittori[1,2*]

[1] *Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Piso 4, Ciudad Universitaria, Ciudad Autónoma de Buenos Aires, C1428EHA, Argentina*

[2] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Autónoma de Buenos Aires, Argentina*

[3] *División Hematología Clínica, Departamento de Medicina, Hospital Nacional Alejandro Posadas, Buenos Aires, Argentina*

ABSTRACT

The widespread use of aluminum (Al) provides easy exposure of humans to the metal and its accumulation remains a potential problem. In vivo and in vitro assays have associated Al overload with anemia. To better understand the mechanisms by which Al affects human erythrocytes, morphological and biochemical changes were analyzed after long-term treatment using an in vitro model. The appearance of erythrocytes with abnormal shapes suggested metal interaction with cell surface, supported by the fact that high amounts of Al attached to cell membrane. Long-term incubation of human erythrocytes with Al induced signs of premature erythrocyte death (eryptosis), such as phosphatidylserine externalization, increased intracellular calcium, and band 3 degradation. Signs of oxidative stress, such as significant increase in reactive oxygen species in parallel with decrease in the amount of reduced glutathione, were also observed. These oxidative effects were completely prevented by the antioxidant *N*-acetylcysteine. Interestingly, erythrocytes were also protected from the prooxidative action of Al by the presence of erythropoietin (EPO). In conclusion, results provide evidence that chronic Al exposure may lead to biochemical and morphological alterations similar to those shown in eryptosis induced by oxidant compounds in human erythrocytes. The antieryptotic effect of EPO may contribute to enhance the knowledge of its physiological role on erythroid cells. Irrespective of the antioxidant mechanism, this property of EPO, shown in this model of Al exposure, let us suggest potential benefits by EPO treatment of patients with anemia associated to altered redox environment. J. Cell. Biochem. 113: 1581–1589, 2012.

KEY WORDS: ALUMINUM; ERYTHROCYTES; OXIDATIVE STRESS; ERYTHROPOIETIN

Exposure to toxic metals is a well-known problem in industrialized countries. Metals interfere with a number of physiological processes, including central nervous system, hematopoietic, hepatic, and renal functions.

The fact that aluminum (Al) is a toxic metal to living organisms, including human beings, was discovered a long time ago. This metal is extensively used in daily life and has multiple applications. It can be found in water and even in food through additives or by contact with packaging, containers, aluminum foil, and kitchen utensils. Moreover, certain pharmaceutical preparations such as antacids are a significant source of Al for humans [Soni et al., 2001]. Therefore, its accumulation may produce negative physiological effects since it has no known essential role within the body.

Even when Al can reach and accumulate in almost every organ in the human body, the central nervous system is a particular target of the deleterious effects of the metal. However, it is known that there is a causal role for Al in other anomalies, such as microcytic anemia and osteomalacia. Al toxicity continues to be a problem among patients with chronic disease under dialysis treatment [Bohrer et al., 2009; Kan et al., 2010]. Nowadays, other population at risk of Al overload has been identified. Clear links have been established between toxicity in infants and parenteral exposure to Al [Burrell and Exley, 2010] since the metal would be present as a contaminant

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*Correspondence to: Dra. Daniela C. Vittori, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Pabellón II, Piso 4, Ciudad Universitaria, (C1428EHA) Ciudad Autónoma de Buenos Aires, Argentina. E-mail: dvittori@qb.fcen.uba.ar

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in formulas received by preterm neonates [Bohrer et al., 2010]. In spite of the numerous efforts and the accumulated evidence in these areas of research, the mechanisms of Al toxicity have not been completely elucidated yet.

Al overload has been associated with signs of anemia [Elliot and McDougall, 1978; O'Hare and Murnaghan, 1982; Wills and Savory, 1983; Touam et al., 1983; Eschbach and Adamson, 1985; Altmann et al., 1988; Abreo et al., 1989; Sedman, 1992; Becaria et al., 2002]. Erythropoiesis may be blocked through mechanisms that involve the interference of Al with iron homeostasis [Pérez et al., 1999, 2001, 2005; Vittori et al., 1999], changes in the enzymes of hem biosynthesis [Chmielnicka et al., 1994; Schoeder and Caspers, 1996], and/or the dysregulation of erythropoietin receptor expression [Vittori et al., 2005]. In previous works, we have demonstrated that not only erythroid progenitors but also mature erythrocytes may be affected by Al exposure. In vitro and in vivo assays showed inhibition of bone marrow colony forming units-erythroid (CFU-E) growth as well as morphologic alterations in mature erythrocytes living in an Al rich-environment. Severe morphological changes were induced by Al, and traces of the metal were detected inside cells with abnormal shape [Vittori et al., 1999] or attached to the erythrocyte membrane [Vittori et al., 2002]. Rats with normal renal function receiving chronic treatment with Al salts in drinking water showed signs which supported the development of anemia [Garbossa et al., 1998a; Vittori et al., 1999]. The results let us suggest that Al may accelerate the progression of eryptosis—erythrocyte premature self-destruction—showing changes that mimic apoptotic features in nucleated cells [Daugas et al., 2001].

We have developed an in vitro model of human erythrocytes which after long-term exposure to Al [Vittori et al., 2002] resemble morphological changes and membrane protein alterations as those observed in red blood cells of rats receiving chronic oral Al overload [Vittori et al., 1999]. In the present work we used this in vitro model to further investigate whether Al exposure may induce eryptosis and elucidate the mechanisms involved.

Considering that erythrocytes of chronic hemodialysed patients seemed to be less susceptible to eryptosis after treatment with recombinant human erythropoietin [Myssina et al., 2003], it was also interesting to study whether the hormone was capable of preventing the Al-induced erythrocyte damage.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

All chemicals used were of analytical grade. 199 Medium was obtained from GIBCO, Penicillin-streptomycin (PAA Laboratories GmbH) were purchased from GENSA. Mouse monoclonal anti-band 3 antibody, sodium o-vanadate, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), *N*-acetylcysteine (NAC) and Mercury Orange were obtained from Sigma–Aldrich. Fluo-4/acetomethylester (Fluo-4 AM) was from Invitrogen Life Technologies. Annexin V-FITC apoptosis detection Kit II was purchased from BD Biosciences. Dual Color Protein Loading Buffer (Fermentas Life Sciences) was from Tecnolab. Miniprotean TGX 4–15% gradient polyacrylamide–sodium dodecyl sulfate (SDS) gels were obtained from Bio-Rad. Chemiluminiscent system kit (ECL), nitrocellulose membranes (Hybond-ECL), and anti-mouse horseradish peroxidase-conjugated antibody were obtained from GE Healthcare Life Sciences. SDS, Triton X-100, Folin-Ciocalteu's reagent, Tween 20, and dimethyl sulfoxide (DMSO) were from Merck. Mouse monoclonal anti-β-actin was purchased from Santa Cruz. Recombinant human erythropoietin (rhEPO) was provided by Zelltek (Argentina).

ERYTHROCYTE ISOLATION

Heparinized human peripheral blood was obtained from healthy donors (25–38 years old) following informed consent. After centrifugation for 15 min at 500*g* and 4°C, plasma and buffy coat were carefully removed, and erythrocytes were washed three times with isotonic phosphate-buffered saline (PBS: 0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) [Vittori et al., 1999].

EXPERIMENTAL MODEL

Aluminum stock solution was prepared immediately before use by dissolving $AlCl_3{\cdot}6H_2O$ in 0.1 N Tris–HCl buffer pH 7.3 at the concentration of 10 mM, and the stock solution was diluted into desired working concentration based on our previous toxicity experiments on mature erythrocytes [Vittori et al., 2002]. All solutions were sterilized through 0.22 μm syringe filters immediately after preparation.

As we mentioned above, in a previous work we found that rats receiving Al in the drinking water for 8 months developed anemia which apart from changes in hematological parameters showed novel erythrocyte morphological alterations [Vittori et al., 1999]. We then developed an in vitro model [Vittori et al., 2002]. Nonphysiological temperature (4°C) was needed to develop long-term assays. Under this condition, erythrocytes exposed to high Al concentrations in a period of 21 days mimic cell changes observed in the in vivo experiments. This in vitro model was used in the present work to continue studying mechanisms involved in the action of Al. Briefly, fresh red blood cells were washed and suspended in 199 Medium containing 1 g/L glucose and supplemented with 100 U/ml penicillin–100 μg/ml streptomycin at 20% hematocrit and treated with 100 μM $AlCl_3$ for 21 days at 4°C. This synthetic medium with chemically known composition allows cell survival without need of serum [Morgan et al., 1950]. It was employed to mimic the environment of circulating erythrocyte.

Control experiments were carried out in a noncomplex solution (HEPES buffer: 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2.5 mM $CaCl_2$, 10 mM glucose, and 0.1% (w/v) BSA, pH 7.4) and in calcium-free HEPES buffer. In addition, erythrocytes were assayed in Al-free medium or treated with 100 μM NaCl, as controls.

When 5 mM NAC or 10 U/ml erythropoietin (EPO) were used, they were added to cells suspensions 30 min before the addition of $AlCl_3$. Medium renewal with or without $AlCl_3$, NAC, or EPO was performed every 2 days. To evaluate oxidant susceptibility, following $AlCl_3$ exposure for 21 days, red blood cells were washed for Al removal and incubated with 1 mM H_2O_2 + 5 mM $NaNO_2$ for 90 min at 37°C.

ASSESSMENT OF CELLULAR MORPHOLOGY BY SCANNING ELECTRON MICROSCOPY

Samples were prepared as previously reported [Vittori et al., 1999]. Briefly, after exposure to each treatment, red blood cells were seeded on a glass support (50 mm^2 area), and fixed for 20 min with 3% (v/v) glutardialdehyde in 0.1 M phosphate buffer, pH 7.4. Samples were washed three times in the same buffer, subsequently dehydrated through successive washes in graded acetone (from 25% to 100%, v/v), and dried directly from acetone in a Balzers CPD 030 Critical Point Bomb using carbon dioxide as a transition fluid. Samples were then coated with a thin layer of gold (Balzers Union SCD 040) and examined using a scanning electron microscope (ZEISS Supra 40).

PHOSPHATIDYLSERINE TRANSLOCATION

Annexin V-FITC is able to label the externalized phosphatidylserine (PS) on the outer layer of plasma membrane. Erythrocytes were suspended in 100 μl of binding buffer (0.01 M HEPES/NaOH pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) at 1% hematocrit. According to manufacturer's instructions, cells were loaded with annexin V-FITC (5 μl) for 15 min at room temperature in the dark. After addition of 400 μl of the binding buffer, cells were analyzed by flow cytometry (FACSort, Becton Dickinson) using excitation at 488 nm. Data were analyzed with the software WinMDI 2.9.

INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

A volume of 250 μl of erythrocytes suspended in PBS at 1% hematocrit was incubated with 400 μM DCFH-DA for 15 min at 37°C in an atmosphere containing 5% CO_2. Following the addition of 400 μl of PBS, cells were analyzed by flow cytometry [Ghoti et al., 2007].

INTRACELLULAR REDUCED GLUTATHIONE CONTENT (GSH)

A volume of 100 μl of erythrocytes suspended in PBS at 1% hematocrit were incubated with 40 μM Mercury Orange for 5 min at 0°C and immediately analyzed by flow cytometry [Ghoti et al., 2007].

INTRACELLULAR CALCIUM LEVEL

After treatment, erythrocytes were collected by centrifugation and loaded with 2.5 μM Fluo-4 AM at 25°C for 30 min according to manufacturer's instructions. This colorless calcium indicator enters the cell freely and is hydrolyzed by nonspecific intracellular esterases to yield fluorescence when bound to free calcium ions. After washing, cells were incubated in PBS for 45 min and then analyzed by flow cytometry using excitation at 488 nm.

ERYTHROCYTE MEMBRANE PREPARATION

The whole procedure was performed at 4°C. Erythrocytes were hemolysed by diluting 1:10 with hypotonic 5 mM sodium phosphate buffer, pH 8.0, containing 50 μM PMSF, 50 mg/L SBTI, and 1 mM sodium o-vanadate. Erythrocytes ghosts and supernatant cytosol fractions were obtained after 30 min centrifugation at 13,000*g*. Pelleted membranes were repeatedly washed until creamy white. Ghosts were suspended in the lysis buffer. Membrane suspensions and cytosol fractions were kept at −70°C [Vittori et al., 1999]. Total proteins were quantified by the Lowry's method [Lowry et al., 1951].

ELECTROPHORESIS AND WESTERN BLOTTING

Western blotting was carried out following the methodology described by Vittori et al. [2002] with modifications. Briefly, membrane proteins were boiled for 4 min in the Dual Color Protein loading buffer, resolved by SDS–polyacrylamide gel electrophoresis (4–15% gradient), and electroblotted onto a nitrocellulose membrane during 1.5 h (transfer buffer: 25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, and 20% (v/v) methanol). Residual binding sites were blocked by 1 h-incubation in Tris buffer saline (TBS: 25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween 20 and 0.5% skim-milk powder, and then membranes were incubated with appropriate concentrations of the specific antibody. After washing with TBS–Tween 20, the immunoblots were probed with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1,000) for 1 h at 20°C and washed. Specific antibody signals were detected using the enhanced chemiluminescence system ECL kit and Fujifilm Intelligent Dark Box II equipment (Fuji) coupled to a LAS-1000 digital camera.

QUANTIFICATION OF ALUMINUM IONS

In order to avoid Al trace contamination, all the plasticware used was immersed in 30% HCl for 1–2 h and then exhaustively rinsed with ultrapure water. Al levels were measured in erythrocyte fractions, working solutions, culture media, and ultrapure water using an atomic absorption spectrometer (Shimadzu AA-6501, Japan) coupled to a graphite furnace atomizer (Shimadzu GFA-6000, Japan) with autosampler. The Al quantified in membrane and cytosol fractions after erythrocyte lysis, was expressed per 10^6 erythrocytes [Garbossa et al., 1998b].

STATISTICS

Results are expressed as mean ± standard error (Mean ± SEM). Comparison among groups was carried out by the Kruskal–Wallis test one-way analysis of variance and the Mann–Whitney *U*-test when corresponding. Least significant difference with $P < 0.05$ was considered as the criterion for statistical significance.

RESULTS

SIGNS OF ERYPTOSIS INDUCED BY ALUMINUM

Previous in vivo and in vitro experiments carried out in our laboratory showed morphological changes and membrane protein alterations in erythrocytes exposed to Al for long periods [Vittori et al., 1999, 2002]. Therefore, we decided to study possible associations between these changes and membrane phospholipid disturbance.

Erythrocytes were incubated with Al chloride to analyze the effect of the metal on cell morphology. Using scanning electron microscopy, we observed that erythrocyte shapes were slightly affected by the aging process (Fig. 1A). Except for a few crenated cells, none of the samples incubated without Al showed morphological changes (Fig. 1A.a). On the other hand, according to our previous results and to Zatta et al. [1989], erythrocytes incubated with Al showed many abnormal shapes. Typical biconcave erythrocytes turned into acanthocytes and stomatocytes, spiculated and cup-shaped cells, respectively (Fig. 1A.b,c). Both acanthocytes

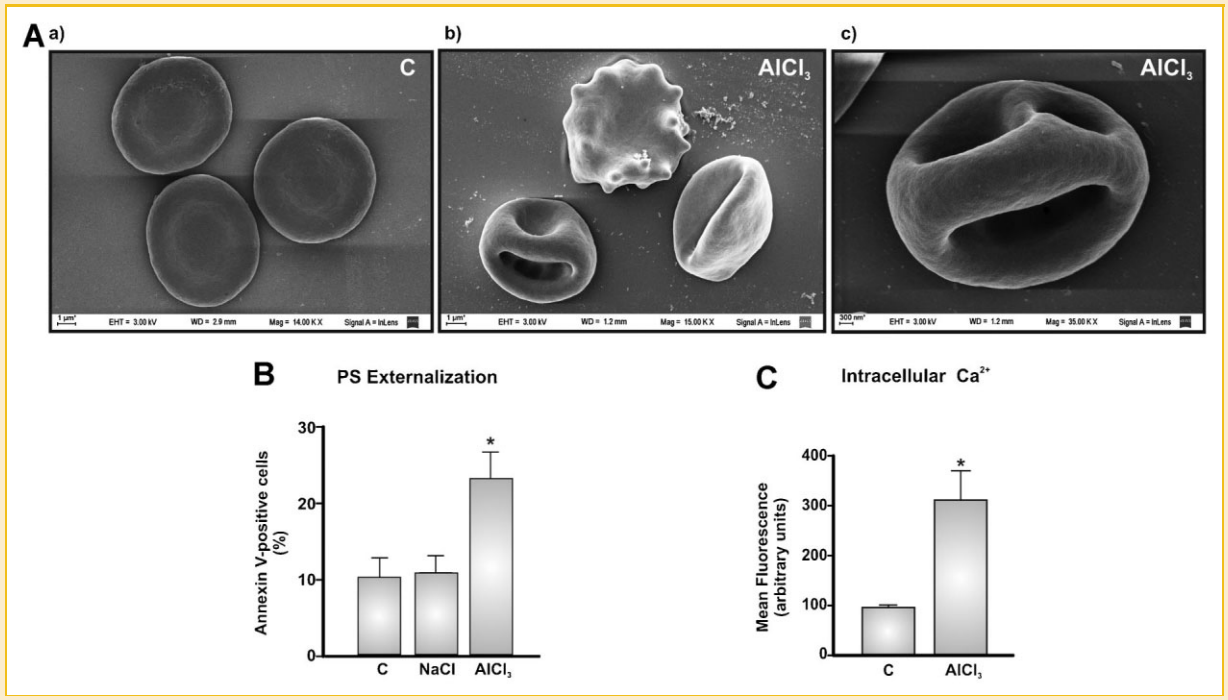

Fig. 1. Programmed cell destruction signs in erythrocytes exposed to aluminum. Red blood cells were incubated in the presence of 100 μM $AlCl_3$ (Al) or in the presence or the absence of 100 μM NaCl as controls (C). A: Following Al exposure, morphological changes were detected by scanning electron microscopy: control erythrocytes, 14,000× (a); Al-treated erythrocytes: acanthocytes and stomatocytes 15,000× (b) and 35,000× (c). B: PS translocation was measured by flow cytometry. Each bar indicates percentage of annexin V positive erythrocytes (Mean ± SEM). C: Following Al exposure, erythrocytes were incubated for 24 h at 37°C, and then loaded with Fluo-4 AM to measure intracellular calcium content by flow cytometry. Each bar indicates geometric mean values of fluorescence (Mean ± SEM). *Significant differences with respect to C ($P < 0.05$, n = 4).

and stomatocytes seem to be the predominant shapes induced by the presence of Al.

Further assays were performed to evaluate whether PS translocation might also be induced in erythrocytes treated with Al for long periods. After 21 day-exposure, flow cytometry analysis showed increased annexin V binding with respect to controls (Fig. 1B, $P < 0.05$).

It has been reported that Al is able to disrupt cellular calcium homeostasis and signaling [Mundy and Shafer, 2001]. Since the signs of eryptosis observed could have been a consequence of calcium influx, the amount of intracellular Ca^{2+} was determined in Al-treated red blood cells by flow cytometry using Fluo-4 AM fluorescence. As shown in Figure 1C, a significant increase of intracellular Ca^{2+} was observed after chronic incubation with Al ($P < 0.05$).

Erythrocytes incubated in the presence of sodium chloride, included in all the experiments presented in this article, showed no significant effects of the chloride anion. In addition, assays in a noncomplex solution (HEPES buffer) showed similar results to those observed in assays with 199 Medium (PS externalization: C 6 ± 2.3%, Al 25 ± 1.9%. Intracellular Ca^{2+}: C 101 ± 22 AU, Al 370 ± 34 AU, expressed as % of Annexin-positive cells and arbitrary units of geometric mean fluorescence, respectively).

In addition, erythrocyte changes were accompanied by threefold increase in hemolysis in the samples treated with Al with respect to controls (data not shown). However, this effect was not attributed to long-term incubation (21 days) because slight but no significant hemolysis was detected in controls incubated during the same period with respect to nonincubated cells.

CELLULAR ALUMINUM CONTENT

In order to confirm the presence of Al inside the cells, quantitative analysis was performed by atomic absorption spectrometry. Al concentrations were determined in membrane and cytosol fractions after erythrocyte lysis. Results showed that Al does enter the cell. After long-term erythrocyte aging in the presence of Al, intracellular as well as membrane Al contents significantly increased when compared to controls (Membrane fraction: C 0.03 ± 0.002; Al 0.47 ± 0.03 ng Al/10^6 erythrocytes. Cytosol fraction: C 0.61 ± 0.08; Al 6.19 ± 0.50 ng Al/10^6 erythrocytes, $P < 0.05$, n = 3). Comparison of Al/C ratios suggest that Al is especially attached to cell membrane.

CELL OXIDATIVE STATUS AFTER ALUMINUM EXPOSURE

Evidence suggests that Al can potentiate oxidative events in different tissues. Furthermore, it is known that cell oxidative stress is one of the causes of premature cell death [Matarrese et al., 2005]. Significant changes in oxidative parameters were observed in erythrocytes exposed to Al for 21 days. Flow cytometry showed a threefold increase in ROS formation in Al-treated erythrocytes (Fig. 2A, $P < 0.05$). Conversely, levels of GSH, a major intracellular defence against oxidative events, significantly decreased (Fig. 2B, $P < 0.05$) when compared to controls.

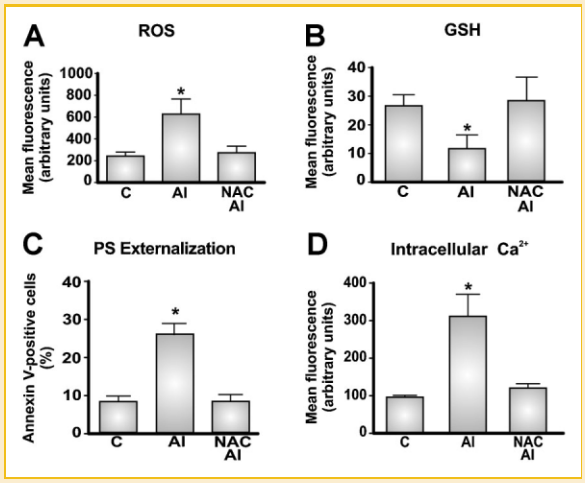

Fig. 2. Changes in erythrocyte oxidative status due to aluminum. Erythrocytes were exposed or not to 100 μM Al during 21 days. Parallel assays were performed in the presence of 5 mM *N*-acetylcisteine (NAC). ROS (A) and GSH (B) were measured by flow cytometry through reactions with 2′,7′-dichlorofluorescin and Mercury Orange, respectively. PS translocation (C) and intracellular calcium level (D) were analyzed by flow cytometry in erythrocytes incubated in the presence of Al or NAC + Al as described in Figure 1. Each bar indicates percentage of annexin V positive erythrocytes (C) or geometric mean fluorescence (A, B, and D) (Mean ± SEM). *Significant differences with respect to C and NAC + Al ($P < 0.05$, n = 3).

To clarify the pathway triggered by Al, assays in the absence of Ca^{2+} were performed. As expected, no increased intracellular calcium content measured by flow cytometry was observed in Al-treated cells (C 103.5 ± 11.6 AU, Al 102.0 ± 11.4 AU, expressed as arbitrary units of geometric mean fluorescence). However, ROS levels and PS translocation were significantly induced by Al even though the latter did not reach the levels observed in the presence of extracellular Ca^{2+} (PS externalization: C 8.4 ± 1.5%, Al 15.4 ± 0.9%. ROS: C 264 ± 19 AU, Al 490 ± 28 AU, expressed as % of annexin-positive cells and arbitrary units of geometric mean fluorescence, respectively). These results suggest that independently of intracellular calcium increase Al is able to induce PS scrambling via its oxidant effect.

RELATIONSHIP BETWEEN SIGNS OF ERYPTOSIS AND OXIDATIVE STRESS

It was interesting to study whether PS translocation and increased intracellular calcium content may be associated to the altered cell oxidative status induced by Al. Experiments were carried out to compare the Al behavior with the effect of known oxidant agents ($H_2O_2 + NaNO_2$, 24 h, 37°C). Similar results as those observed in the presence of Al, such as high erythrocyte ROS levels, increased PS translocation, and calcium intracellular content, were observed under this oxidant environment (data not shown).

To confirm whether Al exerts a prooxidative effect, parallel assays of erythrocytes chronically exposed to the metal were carried out in the presence of NAC. This sulfur-containing compound is one of the antioxidants most frequently used in vitro since the thiol groups are able to reduce free radicals. As seen in Figure 2, the effect of Al was significantly counteracted by the presence of the antioxidant.

To evaluate whether the signs of premature cell destruction in erythrocytes described above could be explained by an oxidative environment produced by Al, PS externalization (Fig. 2C), and intracellular calcium (Fig. 2D) were measured in the presence of NAC and Al. The antioxidant also proved to prevent these signs of eryptosis.

DEGRADATION OF MEMBRANE PROTEIN BAND 3 AFTER LONG-TERM ALUMINUM INCUBATION

As we have previously reported, band 3 degradation was observed after human erythrocytes were incubated in Al-rich medium [Vittori et al., 2002]. This was detected by increase in the concentration of low molecular mass fragments, particularly circa 40 kDa. Protein breakdown was also prevented when erythrocytes were simultaneously incubated with Al and antioxidant NAC (Fig. 3).

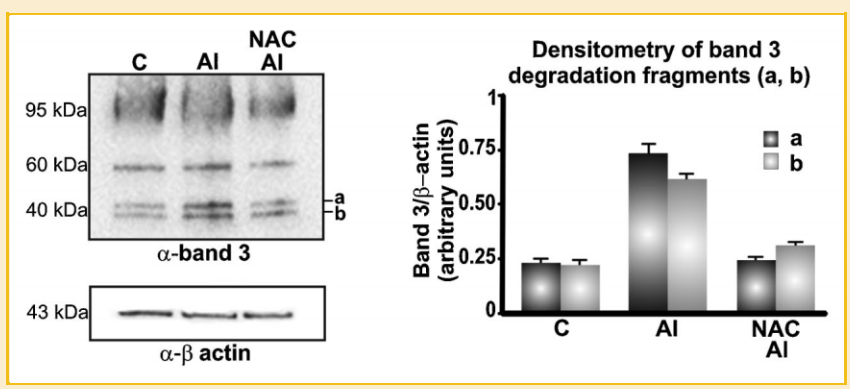

Fig. 3. Effect of aluminum on erythrocyte membrane protein band 3. Isolated membrane proteins from erythrocytes exposed to 100 μM Al or 5 mM NAC + 100 μM Al were resolved in a 4–15% gradient SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Band 3 and β-actin were detected by immunoblotting. Near 40 kDa low molecular mass fragments of band 3 (a, b) are identified. Bars represent density ratio of degraded band 3 (a, b) with respect to actin. The figure is representative of 3 independent assays with similar results.

SUSCEPTIBILITY OF AL-EXPOSED ERYTHROCYTES TO OXIDATIVE STRESS

Evidence demonstrates that osmotic shock, oxidative stress, and calcium accumulation are among the most potent stimuli to induce eryptosis [Matarrese et al., 2005; Lang et al., 2006]. Based on the results described above, it was interesting to study whether erythrocytes previously exposed to Al become more sensitive to additional oxidative events. Erythrocytes were incubated in the presence or the absence of Al for 21 days, and after Al removal, they were exposed to sodium nitrite plus hydrogen peroxide for 90 min. Then, annexin V binding (Fig. 4A), intracellular calcium (Fig. 4B), and ROS levels (Fig. 4C) were measured by flow cytometry. Results show that after oxidative stress, the fluorescence corresponding to PS translocation, intracellular ROS generation and calcium content significantly increased ($P < 0.05$) in erythrocytes previously exposed to Al for long periods.

EFFECT OF ERYTHROPOIETIN

As abovementioned, no physiological role has been described for EPO on mature red blood cells. However, an association between EPO treatment and erythrocyte susceptibility to hyperosmotic shock has been observed in chronic hemodialysed patients [Myssina et al., 2003]. Therefore, we investigated whether the presence of EPO would be able to counteract the deleterious effects caused on erythrocytes by long-term exposure to Al. As can be seen in Figure 5, the presence of EPO during Al exposure avoided ROS production (Fig. 5A), and in addition, PS translocation (Fig. 5B), and calcium influx (Fig. 5C) returned to control values.

DISCUSSION

The experimental in vitro model of human erythrocytes chronically exposed to Al used in this work was previously developed to resemble the toxic effects induced in rats by the ingestion of Al [Vittori et al., 1999, 2002]. The Al dose and incubation period used in this model, required to get cellular changes similar to that ascribed to in vivo Al effects, support realistic in vitro prediction of toxicity.

Present results provide evidence that long-term treatment with Al induce a disruption of the oxidative cellular status with appearance of characteristic features of eryptosis.

Erythrocyte cell membrane scrambling with subsequent PS translocation had been previously reported after 24 h of Al exposure [Niemoeller et al., 2006]. However, different mechanisms seem to be involved in acute and chronic Al intoxication. Oxidative stress is not likely involved in the activation of eryptosis after short Al exposure [Niemoeller et al., 2006]. Instead, here we demonstrate an oxidative role of Al in programmed erythrocyte death following chronic treatment resembling the effects of oxidative compounds such as hydrogen peroxide and sodium nitrite. According to our results, Al action appears to be linked to oxidative stress in other tissues [Bondy and Campbell, 2001].

Phosphorylation and dephosphorylation of protein tyrosine residues is involved in the regulation of several erythrocyte functions. In this context, it has been reported that erythrocyte band 3 phosphorylation is one of the signs observed during oxidative/inflammatory processes [Metere et al., 2009; Bordin et al., 2010]. Since protein phosphorylation and dephosphorylation are dynamic events, we were unable to investigate band 3 phosphorylation at the end of a chronic experience, such as the present model of Al toxicity. Instead, we observed band 3 protein degradation after erythrocyte exposure to Al. In accordance, it has been proposed that degradation of band 3 protein is involved in the generation of a senescence signal of erythrocytes that leads to the identification and removal of old erythrocytes from the circulation [Kay et al., 1989].

We have already reported the sensitivity to Al showed by erythroid cell populations of animals chronically exposed to the

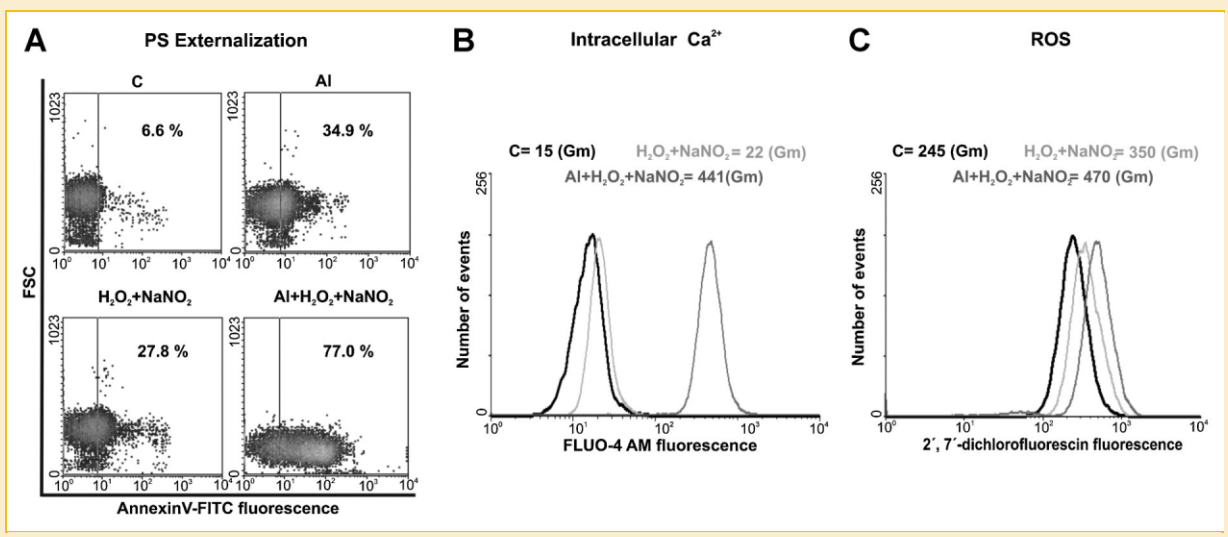

Fig. 4. Susceptibility of Al-exposed erythrocytes to oxidant agents. Following 100 μM Al exposure, and after its removal, erythrocytes were incubated in the presence of $H_2O_2 + NaNO_2$ for 90 min at 37°C. Immediately after, PS translocation (A), intracellular calcium (B), and ROS (C) levels were measured by flow cytometry. Results are expressed as percentage of annexin V positive cells (A) or histograms representing geometric mean values of fluorescence intensity (B,C). The figure is representative of 3 independent assays with similar results.

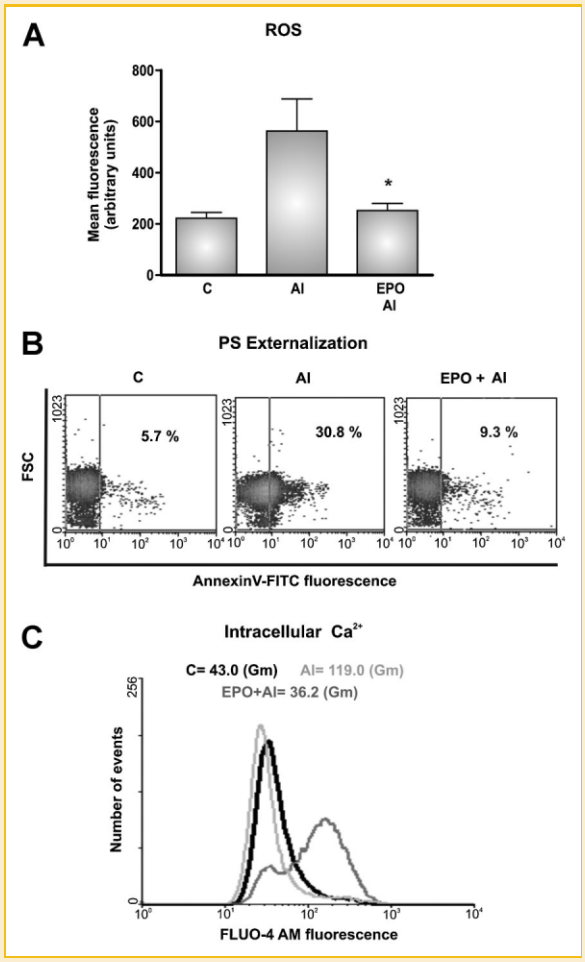

Fig. 5. Protective effect of erythropoietin on erythrocytes incubated with aluminum. EPO (10 U/ml) was added to erythrocyte suspensions 30 min before the addition of 100 μM Al (EPO-Al). After the incubation period (21 days), erythrocytes were subjected to flow cytometry analysis. ROS (A), PS translocation (B), and intracellular calcium level (C) were determined as described in Figure 1. Results are expressed as percentage of geometric mean values of fluorescence intensity (Mean ± SEM; $P < 0.05$, $n = 3$) (A), percentage of annexin V positive cells (B), or histograms representing geometric mean values of fluorescence intensity (C). The figure is representative of three independent assays with similar results.

metal with loss of erythrocyte typical biconcave shapes, with the appearance of acanthocytes and stomatocytes [Vittori et al., 1999; Nesse and Garbossa, 2001]. In the current study, these morphological alterations were found in parallel with significant increases of PS exposure in the external leaflet of membrane bilayer. According to Suwalsky et al. [1999], morphological changes induced by Al suggest that the metal interacts with both the outer and the inner membrane layers. The fact that high amounts of Al entered the cell and attached to the membrane supports the interaction of Al with human erythrocyte membrane.

Other mechanisms such as lipid peroxidation and/or translocase activation may contribute to membrane phospholipid externalization. It has been proposed that metals without redox capacity such as Al can make fatty acids more available to be attacked by free radicals, thus facilitating the propagation of lipid peroxidation [Oteiza et al., 1993; Ohyashiki et al., 1998]. Oxidative stress in cells occurs when the production of free radicals overwhelms the antioxidant defence system. After Al exposure, significant increases in ROS levels were observed in parallel with decreases in the amount of GSH, the main antioxidant defensive compound in the cell.

It has been reported that lipids collapse due to the activation of phospholipid scramblase, which catalyzes the bidirectional transbilayer movement of the major phospholipid classes [Bevers and Williamson, 2010]. The protein involved in this activity is still unknown but multiple pathways are thought to converge on scramblase function. In this regard, there is little doubt that the calcium activation pathway may be physiologically relevant. Indeed, the significantly higher intracellular Ca^{2+} content detected in erythrocytes after incubation with Al suggests that this activation pathway cannot be dismissed. In a previous work, erythrocyte Ca^{2+} level also increased after 6 h of Al treatment, but 24 h-exposure did not reduce erythrocyte GSH content [Niemoeller et al., 2006]. According to our results, it seems that chronic exposure to the metal would be necessary to reach a concentration of free radicals able to disrupt the oxidative system. Other in vitro model showed that lead incubation also produced increased intracellular Ca^{2+} dependent on the erythrocyte oxidative status [Quintanar-Escorza et al., 2010].

Regardless of the mechanisms involved, assays carried out in the presence of antioxidant NAC confirmed the prooxidant damage exerted by Al upon human erythrocytes. Simultaneous treatment of red blood cells with NAC effectively protected cells from Al toxicity avoiding intracellular GSH depletion. NAC also effectively inhibited ROS generation and intracellular calcium accumulation induced by long-term incubation with Al. Furthermore, NAC prevented PS externalization, the main sign of cell eryptosis. Results suggest that intracellular ROS production may be involved in the onset of Al toxicity while GSH may play an important role in the protection of cell viability. It is not surprising that this reduced protection against oxidative stress significantly enhanced cell susceptibility to an extra oxidative environment. Despite Al removal, erythrocytes chronically exposed to Al were more sensitive to a short treatment with oxidant agents than controls. This has to be taken into account especially when Al accumulation occurs in pathologies involving oxidative damage such as chronic kidney disease.

In the present in vitro model, no significant hemolysis was detected due to long period erythrocyte incubation. It has to be noted that PS exposure on cell surface may signal the sequestration of red cells from circulation carried out by macrophages. It is expected that due to an in vivo proeryptotic action of Al, erythrocytes may turn into anomalous cells more prone to be eliminated by the reticuloendothelial system. As expected, lack of these cells in our in vitro model led to increased erythrocyte hemolysis. This would lead to premature erythrocyte death, reducing red blood cell survival, and consequently, causing anemia to develop and/or be aggravated.

Mature erythrocytes from patients with chronic renal insufficiency exhibited higher annexin binding when compared with red blood cells from healthy individuals [Bonomini et al., 1999]. Moreover, Myssina et al. (2003) observed that the number of annexin binding cells decreased after dialysis only when patients

received EPO immediately before dialysis. These authors demonstrated that EPO inhibits the cation channel, thus interfering directly with erythrocyte apoptosis. On the other hand, Chattopadhyay et al. [2000] proposed a different mechanism for EPO action according to which EPO would protect red cell membranes from lipid peroxidation by scavenging hydroxyl radicals generated by the treatment of red blood cells with copper ascorbate. In this regard, our results suggest that EPO has an antioxidant function.

In conclusion, oxidative stress is involved in activation pathways triggered by Al, which may account for the morphologic and biochemical changes in erythrocytes chronically exposed to the metal in vitro. Decreased GSH and increased ROS seem to be responsible for calcium homeostasis disruption and make erythrocytes potentially susceptible to other damaging events. The activation of enzyme-mediated pathways causing oxidative modifications of lipids and degradation of proteins may destabilize membrane skeleton with the appearance of morphologic alterations and membrane scrambling, typical signs of eryptosis. The fact that the Al effects could be prevented by the presence of an antioxidant compound is a strong argument for the prooxidant ability of this metal. Recently, the report of the first computational evidence of a strong likelihood of the formation of an aluminum superoxide semireduced radical anion in physioplogical media [Mujika et al., 2011], reinforces the idea that the presence of Al in biological systems could lead to an important prooxidant activity through a superoxide formation mechanism.

Even though more investigation is needed for further clarification, the interesting finding of the antieryptotic effect of EPO may contribute to enhance the knowledge of its physiological role on erythroid cells. Irrespective of its mechanism of action, the antioxidant property of EPO, shown in this model of Al exposure, let us suggest potential benefits by EPO treatment of patients with anemia associated to altered redox environment.

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