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Original Communication

Use of RNA content to identify reticulocytes in the *in vivo* micronucleus test in humans

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ABSTRACT

Flow cytometric enumeration of micronuclei in the *in vivo* micronucleus (MN) test in rodents is an accepted, well validated test in use all over the world. However, its validation for use in human samples is still incomplete due to the very low number of immature reticulocytes carrying the transferrin receptor CD71 in human blood and due to the great variability that they show in response to medical treatments and exposure to environmental chemicals. Our study is a proposal of a flow cytometric method for enumerating micronucleated reticulocytes in human peripheral blood, based on the classical identification of cytoplasmic content of RNA using thiazole orange. It is demonstrated that the use of this dye in combination with Hoechst 33342 to identify DNA allows a robust calculation of MN frequency in microliter quantities of blood samples from children and young adults.

KEYWORDS: micronuclei, peripheral blood, human reticulocytes

INTRODUCTION

The micronucleus (MN) test in peripheral reticulocytes has been proved to be a sensitive and useful test to evaluate genotoxic damage by agents that are capable of reaching bone marrow and damaging precursor cells.

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The enumeration of MN in polychromatic erythrocytes (PCE) in bone marrow by using fluorescent dyes was first proposed by Hayashi *et al.* [1], who devised a system that uses a differential staining with acridine orange to evaluate under a fluorescence microscope. Normochromatic erythrocytes (NCE) would not be stained and young PCE would have their cytoplasm, abundant in RNA, stained in red; double stranded DNA of MN would be stained in yellowish green, thus allowing the rapid enumeration of the most recently induced damage in the youngest erythrocytes. This analysis was faster and easier than the analysis with Giemsa in a bright field microscope and eliminated some artifacts produced by Giemsa staining. This method was later improved by the use of an ultra-vital staining with slides previously coated with acridine orange, which allowed for a sharper recognition of reticulocytes with abundant RNA in their cytoplasms [2]. In this same publication, they demonstrated that blood could be used to evaluate genotoxic effects of compounds in the *in vivo* micronucleus test.

Collaborative studies further demonstrated that the results were repeatable, reproducible and sensitive to the action of a wide variety of chemicals and different treatment protocols [3, 4], in both mice and rats, even though the spleen in the rat eliminates the defective cells carrying MN. It was demonstrated that it was possible to analyze genotoxic effects of recent exposures by enumerating young RNA-containing reticulocytes. Microscopic evaluation, however, is time-consuming.

Since erythrocytes and reticulocytes lack a nucleus, Grawé *et al*. [5] realized that micronuclei could be easily enumerated by using flow cytometry and described a method to do so with the use of Hoechst 33342 to stain micronuclei containing DNA, and thiazole orange (TO) to stain RNA in the cytoplasm of young reticulocytes in mouse peripheral blood. This method of analysis requires an equipment with two lasers, which provide a range of wavelenghts in the violet to blue regions of the visible spectrum (450 to 640 nm), giving trustable results with high correlation with microscopy determinations of micronuclei, with the advantage that 10,000 cells could be easily evaluated for the presence of micronuclei in a few minutes, compared to 2,000 cells that took much longer to be evaluated under the microscope.

The above-mentioned method proved to be a good substitute for the evaluation under the microscope; however, it requires a dual laser device, which is not usually found in most genetic toxicology laboratories. For this reason, Dertinger *et al*. [6] suggested an alternative method to determine micronuclei in reticulocytes using a single laser device. Technical problems emerged because of the difficulty of accurately differentiating DNA fluorescence emission from that of the RNA using acridine orange; even though DNA emits at 525 nm and RNA emits at 650 nm, reproducibility was not good. This problem was solved by using CD71, the transferrin receptor, as the reticulocyte marker instead of RNA; in this way, an antibody against CD71 conjugated to fluorescein isotiocyanate (FITC) with a fluorescence that could be identified in a different channel would be used to identify reticulocytes, whereas propidium iodide would be used to identify DNA in the micronuclei. The method was carefully documented in several papers [6-10] and became reproducible and transferable for the *in vivo* MN assay in rodents. Inter-laboratory studies demonstrated its utility to evaluate the genotoxicity of known agents [11, 12].

Reticulocytes carrying the transferrin (CD71) receptor constitute the youngest subpopulation of circulating reticulocytes which are the youngest population of erythrocytes in peripheral blood. Their main characteristic is the presence of ribosomes in the cytoplasm that are catabolized in one or two days after being released from the bone marrow into the bloodstream. The CD71 receptor is present in 1-5% reticulocytes, whereas RNA-containing reticulocytes amount to 4-11% [13-15] in peripheral blood of rats. In the manner in which the above-mentioned method has been standardized, there are enough CD71 positive cells to analyze micronuclei, even though some treatments such as those using cis-platin and other antineoplastic drugs [9, 15] as well as radiation therapy [16] greatly reduce their frequency in blood.

When it comes to applying the methodology to human samples, however, the proportions are different. The amount of $CD71^+$ reticulocytes in humans is considerably lower than in rodents, 0.05-0.5% (Table 1) [16, 17]. This difference means that a greater number of cells should be acquired in the cytometer in order to achieve the quantities obtained when using rat samples, i.e., if the percentage of $CD71^+$ cells in rats is 1-5% and in humans is 0.05-0.5%, 10X to 20X more events have to be evaluated in order to obtain the amount of $CD71^+$ cells necessary to estimate the MN frequency, which means that 10 to 20 million events have to be analyzed per human sample. These figures make the analysis time-consuming and increase the risk of artifacts. In practice, the analysis of human samples using CD71 to identify only the youngest reticulocytes falls in the range of the manual evaluation under the microscope [18].

Table 1. Reported frequencies of peripheral blood reticulocytes in rat and in humans.

Marker for identification	Rat (%)	Humans (%)	References
RNA in the cytoplasm	4-11	0.5-3	[14, 15] (rat) [19] (human)
Transferrin receptor CD71*	1-5	0.05-0.5	[13-15] (rat) [16, 17] (human)

*: Reticulocytes carrying the transferrin receptor are the youngest subset of these cells. The receptor is lost very early in the maturation of these cells in the blood [23].

The method of using TO for staining cytoplasmic RNA, first proposed by Grawé *et al*. [5] becomes more attractive, as a greater population of reticulocytes can be evaluated, making it more likely to collect a robust sample to evaluate MN frequency. Total reticulocytes carrying RNA in normal human blood have been reported to range between 0.5% and 3% [19].

Modifications to this methodology, however, were made on the assumption that micronucleated reticulocytes might be removed from the circulating blood by the spleen, and hence events could be lost when analyzing the whole cohort of reticulocytes. The solution proposed by the same group of researchers was to immunologically isolate $CD71^+$ cells first, and then enumerate micronuclei by using their standard cytofluorometric protocol [20].

However, this method not only implies the use of expensive magnetic beads and magnetic device to isolate the cells but also makes the processing of samples more time-consuming and adds the risk of variations during the isolation procedure. It also requires a larger blood sample (at least 2 ml), which could be used for other determinations in human biomonitoring studies.

Owing to the aforementioned reasons, we saw the need to develop a simpler strategy to enumerate MN in human reticulocytes, based on the most universally accepted method due to its accessibility [21] and because it offers the possibility of enumerating this important biomarker in microliter quantities of blood. The purpose of the present paper is to demonstrate that reticulocytes containing cytoplasmic RNA are the population of choice to obtain a robust calculation of the MN frequency in humans.

MATERIALS AND METHODS

The following antibodies were purchased from BioLegend: anti-rat CD71 conjugated to PE/Cy7 (emission at 780 nm, blue laser), anti-rat CD61 conjugated to PE (emission at 575 nm, blue laser), anti-human CD71 conjugated to PE/Cy7, and anti-human CD61 conjugated to PE. Hoechst 33342 (emission at 461 nm, violet laser) and TO (533 nm, blue laser) were purchased from Sigma. BBS (bicarbonate buffer saline) (0.9 g NaCl, 44.4 mg $NaHCO_3$ per 100 ml water) was used to dilute them. For cytofluorometric analysis a dual laser Attune® Acoustic Focusing Cytometer from Applied Biosystems was used, with blue-violet lasers (488 nm and 405 nm, respectively).

Blood samples from 20 normal children aged 8 to 13 years and from 20 adults aged 19 to 31 years were used in the study. Also, blood was used from rats treated with hydroquinone (50 mg/Kg b.w., p.o., n = 5) and from untreated rats (n = 5). Rats were treated once per day for two consecutive days and blood was drawn for analysis 24 hours after the second treatment.

Ethical approval and informed consent

The use of animals and the use of human blood samples were approved by the ethical committees of the Institute of Biomedical Research of the National Autonomous University of Mexico, based on the Ethics Code for Academic Personnel and national and international regulations. Informed consent was obtained from adults and from the parents of the children participating in the study.

Sample fixation

Fixation and part of the staining procedure are mainly based on the protocols by Bemis *et al*. [21] and Litron Labs Rat Flow Manual [22], and staining with TO and Hoechst 33342 was based on Abramsson *et al*. [20].

Rat blood samples were extracted using heparinized capillary tubes; 100 microliters were diluted in 320 microliters of a sodium heparin solution (500 U/ml BBS – 0.9 g NaCl + 0.04 g $NaHCO_3$ in 100 ml distilled water) and gently homogenized. 180 microliters of this sample solution were taken in a pipette and forcefully fixed in 2 ml frozen methanol (previously kept at -80 °C in 5 ml cryotubes, Sarstedt) and mixed in the vortex, capped and mixed again for 5 sec and immediately put back in the ultrafreezer and kept at -80 °C until use.

Human blood samples were taken using heparinized vacutainers (Becton-Dickinson). 250 microliters were diluted in 1.25 ml of sodium heparin solution (500 U/ml BBS) and gently homogenized. 400 microliters of this sample solution were taken in a pipette and forcefully fixed in 4 ml frozen methanol (in 5 ml cryotubes), mixed in the vortex, capped and mixed again for 5 sec and immediately put back in the ultrafreezer at -80 °C until use.

Staining

A solution of BBS was prepared for the staining of nucleic acids. Batch solutions of Hoechst 33342 (500 mM in water) and TO (1 mg/ml in methanol) were used in the following proportions: 7.5 microliters of the former and 2 microliters of the latter were added to 10 ml BBS. An adequate quantity of this solution was prepared depending on the quantity of samples to be stained [20].

Samples were retrieved from the ultrafreezer and maintained at 4 °C. 1 ml of the fixed human sample was transferred to 11 ml of cold BBS with 1% fetal bovine serum (0.5 ml of rat samples was used in 11.5 ml of BBS).

Samples were vortexed for 5 sec and centrifuged at 1,500 rpm for ten minutes at 4 °C. The supernatant was withdrawn, leaving 100 microliters of BBS to re-suspend the cells. 25 microliters were used for the staining procedure in cytometry tubes (Falcon, 5 ml) with 5 microliters of anti-CD71-PE-Cy7 (1 microliter for rat samples), 1 microliter of anti-CD61-PE (the same amount for rat samples) and enough BBS containing 1% fetal calf serum (FCS) made to a final volume of 100 microliters. Henceforth, the same procedure was used with human and rat samples.

Incubation took place for 30 min at 4 °C and 60 min at room temperature (23 °C), after which time 2 ml BBS containing Hoechst 33342 and TO were added. Incubation was done at 37 °C for 60 min, after which time the samples were kept at 4 °C until analysis. One tube per sample was used. Five staining controls were prepared each time: one per fluorochrome and one blank for the compensation procedure. The simultaneous use of these fluorochromes allowed for both the simultaneous determination of CD71 and RNA in reticulocytes and the enumeration of the frequency of each marker in these cells.

Acquisition of samples in the cytometer

An Attune® Acoustic Focusing Cytometer from Applied Biosystems was used with a Blue/Violet: 488 nm (20 mW) and 405 nm (50 mW) lasers configuration and controlled for acquisition and analysis by the Attune® Cytometric Software. Channels BL1-H, BL2-H, BL3-H and VL1-H were used to detect TO, PE, PE-Cy7 and Hoechst 33342, respectively (Table 2). BL1-H (530 nm) and VL1-H (450 nm) wavelengths are sufficiently separated and hence no overlap exists and each one detects the output of different lasers. Adequate controls for each detector were used for automatic compensation by the equipment.

Three gates were used for acquiring 10,000 to 12,000 TO^+ reticulocytes with each sample in triplicate, i.e., a number of 36,000 TO^+ reticulocytes were acquired for each donor (this was accomplished by establishing the volume of sample that is taken for analysis, varying from 200 to 250 microliters in the case of rats, and from 500 to 600 microliters in the case of human samples). Fig. 1 shows the gating done for the acquisition of each sample in the cytometer. The data collected this way were stored together with the settings and the compensation matrix in a file to be analyzed in another computer.

Analysis

The analysis was done using the Attune Cytometric Software. Additional gates were established to eliminate platelets (PE-$CD61^+$ cells) and nucleated cells (Fig. 2), generally following the indications by Bemis *et al.* [21] and Litron Labs Rat Flow Manual [22]; R4 is the amount of $CD61^+$ cells, namely platelets, and R5 is the region where nucleated cells are detected. Derived gates were generated in the following manner: NOT R4 is set to exclude platelets, then NOT R5 is set to exclude nucleated cells, then they are combined to exclude both cell types at the same time [NOT R4 AND NOT R5],

Table 2. Cell markers identified and fluorochromes used for each detector and laser.

Detector	Laser	Fluorochrome	Emission wavelength (nm)	Cell marker
BL1-H	Blue	Thiazole orange (TO)	533	RNA
BL2-H	Blue	Phycoerythrin (PE)	570	CD61
BL3-H	Blue	PE-Cy7	780	CD71 (TfR)
VL1-H	Violet	Hoechst 33342	460	DNA

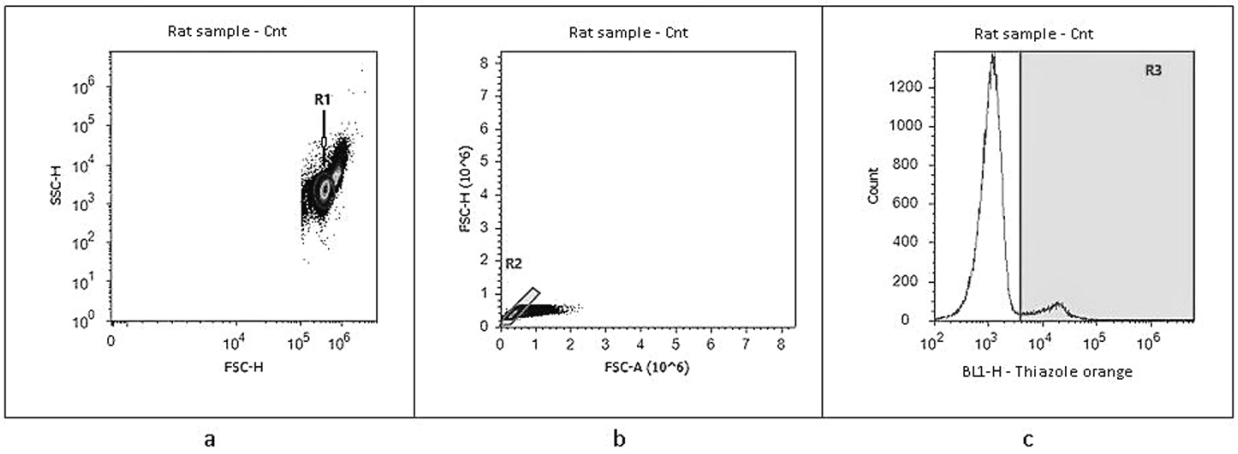

Fig. 1. Gating for the acquisition of 12,000 cells stained with TO. a) Size (FSC-H) vs. granularity (SSC-H) was used to select for single cells (R1, pointed by the arrow). b) Cell population in gate R1 was used to further identify single cells using a new graph representing size in height (FSC-H) vs. size in area (FSC-A), and a new region was depicted and named R2. c) Gate R2 was used to generate this histogram to establish a third gate corresponding to cells positively stained with TO identified by the BL1-H detector; these cells are gated in R3. The instrument was set to acquire 12,000 events in this region. This procedure was done with each sample and data were stored in a file for later analysis.

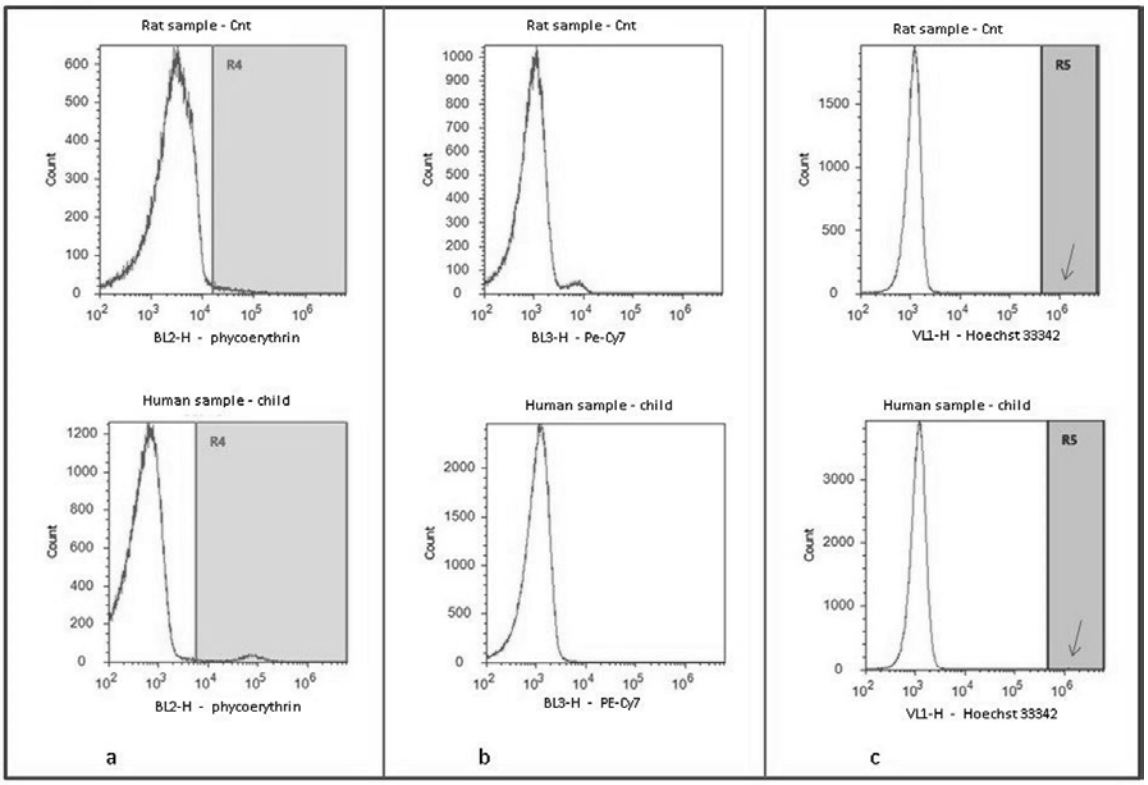

Fig. 2. Additional gating to select the population of interest: erythrocytes. Upper graphs correspond to a rat sample; lower graphs correspond to a human sample. a) These histograms show detector BL2-H, which was used to detect platelets positive to phycoerythrin (PE); they were gated in R4. b) These histograms were used to monitor the presence of CD71 positive cells, stained with Pe-Cy7 and detected in BL3-H. c) Detector VL1-H (violet laser) was used to identify cells stained with Hoechst 33342 for dsDNA, including nucleated cells, signaled by arrows and gated in R5.

and lastly R2 is selected as the population from which micronuclei are evaluated, which corresponds only to erythrocytes [NOT R4 AND NOT R5 AND R2]. This last gate was used in the graphs shown in fig. 3 to obtain the following data: TO^+ cells (reticulocytes) which correspond to R6; TO^+ and $Hoechst^+$ cells (reticulocytes with micronuclei) which correspond to R7; cells negative to both fluorochromes (mature erythrocytes) which correspond to R8 in the lower left quadrant; and cells positive to Hoechst and negative to TO (mature erythrocytes with micronuclei) which are found in R9. A threshold was established with the quadrant in the logarithmic value of 10^4 of graphs VL1-H vs BL1-H to clearly separate negative and weakly positive cells from the most brilliant in the BL1-H channel (Fig. 3, panel a), so as to enumerate only the youngest reticulocytes with more RNA in the cytoplasm and to use this datum to calculate the micronucleus frequency of reticulocytes. There is an intermediate population of cells weakly positive to TO (pointed by arrows in fig. 3, panel a) between this threshold and the negative population, which forms a compact figure in the density graph. These cells are abundant and have been consistently found among different experiments under our staining conditions.

To determine the amount of $CD71^+$ cells with and without micronuclei, a second density graph showing channels VL1-H vs BL3-H was used (Fig. 3 b panel), where regions R10 and R11 were used for evaluation of cells positive to CD71, and negative (R10) or

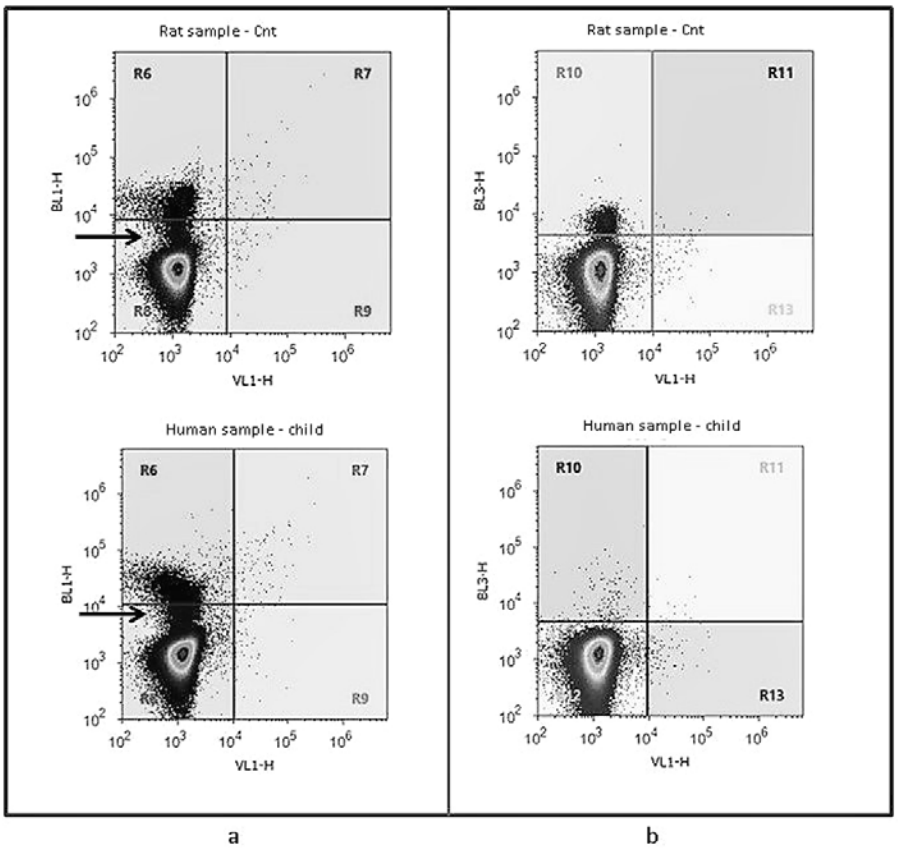

Fig. 3. The derived gate [NOT R4 AND NOT R5 AND R2] was used in these density graphs to enumerate the following: a) cells positive to TO (R6 and R7, detected by BL1-H) and positive to Hoechst (R7 and R9, detected by VL1-H). R8 are cells negative to TO and to Hoechst, corresponding to mature erythrocytes; b) cells carrying CD71 and positive to Pe-Cy7 (R10 and R11, detected by BL3-H) and positive to Hoechst (R11). R12 and R13 are negative to CD71, but many of these cells are positively stained with TO. Graphs in the top show a typical rat sample; graphs in the bottom show a typical human sample. Arrows point to cells weakly stained with TO, which were counted among the mature erythrocytes.

positive (R11) to Hoechst. Cells in R12 and R13 are negative to CD71, but a large amount of them are positive to TO as shown in fig. 4, and can be used to evaluate the micronucleus frequency.

Evaluation and statistical analysis

Each sample was acquired three times, and thus three sets of data were generated for each donor. Data acquired for each region of interest were saved in an Excel sheet for analysis. One sheet each was generated for rats (Fig. 5) and for humans. Data for each donor were added before calculating the reticulocyte and micronucleus frequencies. Then the calculations shown in table 3 were done. The results were used to generate a Stata sheet for statistical analysis, which consisted in comparing the distribution of each category by Kruskal-Wallis test (K-W), establishing a p value of <0.05 for significance. Categories for the rat study consisted of different treatments: negative controls and treatment with hydroquinone. Categories for humans were children and adults.

RESULTS

Measurements in rat samples used as a reference

Table 4 shows the mean values and standard deviations of analyses of blood of untreated rats and rats treated with hydroquinone.

It can be seen how the treatment with hydroquinone reduced the fraction of circulating reticulocytes -

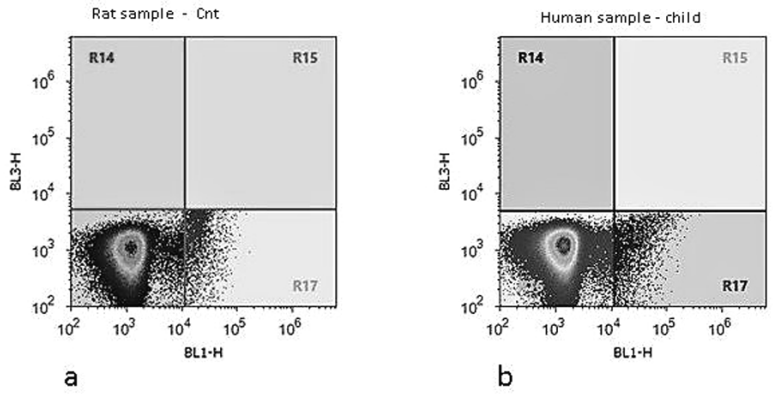

Fig. 4. Cells negative to CD71 in regions R12 and R13 shown in fig. 3 were gated together to analyze them for TO staining (detector BL1-H). It was demonstrated that a large amount of CD71 negative cells (BL3-H) are reticulocytes containing RNA that can be evaluated for the MN frequency: a) rat sample, b) human sample. Region R17 in a) contains more than 5,000 cells; R17 in b) contains 7,195 cells. The quadrant was set to place cells weakly stained with TO in the negative area (R16).

Trtment	Sample	R3	Main gate	R6	R7	R8	R9	R10	R11	R12	R13	Ret TO+
CN	Ctrl 1a	10488	141769	8843	71	132808	47	1949	12	139704	104	8914
CN	Ctrl 1b	10797	148924	8955	84	139810	75	1966	15	146800	143	9039
CN	Ctrl 1c	10609	141263	8838	66	132305	54	1992	10	139154	107	8904
CN	Ctrl 2a	10969	125038	9167	86	115712	73	3100	10	121782	146	9253
CN	Ctrl 2b	11180	126221	9319	96	116748	58	3211	19	122862	129	9415
CN	Ctrl 2c	11093	125902	9249	90	116499	64	3124	13	122629	136	9339
CN	Ctrl 3a	11599	148303	9427	117	138684	75	2773	29	145339	162	9544
CN	Ctrl 3b	11342	144129	9350	95	134604	80	2698	22	141260	149	9445
CN	Ctrl 3c	11777	146256	9556	114	136509	77	2879	24	143188	165	9670

Fig. 5. Figure of an Excel sheet showing the data obtained for each sample, which were used to calculate the percentages of TO^+ and $CD71^+$ reticulocytes, as well as the percentages of micronucleated reticulocytes of each type; also included are micronucleated mature erythrocytes. The regions (R) are explained in fig. 3, except for "Main gate", which refers to the evaluable erythrocyte population defined by [NOT R4 AND NOT R5 AND R2] and "Ret TO^+" which is the sum of R6 plus R7. Notice that each sample was evaluated three times as shown in the shaded area.

Table 3. Parameters determined using the data obtained from the specified regions.

Parameters determined	Specific regions/sub-population evaluated[#]
% TO^+	(Ret TO^+/main gate) x 100
% $CD71^+$	(R10+R11/main gate) x 100
% $CD71^+/TO^+$	(R10+R11/Ret TO^+) x 100
% MN TO^+	(R7/Ret TO^+) x 100 (only TO^+ reticulocytes)
% MN $CD71^+$	(R11/R10+R11) x 100 (only $CD71^+$ reticulocytes)
% MN $CD71^+/TO^+$	(R11/Ret TO^+) x 100 ($CD71^+/TO^+$ reticulocytes)
% MN m.e.	(R9/R8+R9) x 100 (only mature erythrocytes)

[#]: Refers to sub-population determined by the indicated gates or regions. "Main gate" refers to the evaluable cell population of erythrocytes defined by [NOT R4 AND NOT R5 AND R2], see 'Analysis' section. "Ret TO^+" is the whole TO positive population of reticulocytes defined by adding R6 + R7.

Table 4. Results obtained from blood of untreated and treated rats.

Parameters	Untreated (mean % ± s.d.)	Hydroquinone (mean % ± s.d.)	p
% TO^+	7.23 ± 1.0	3.57 ± 0.2	0.0001
% $CD71^+$	1.59 ± 0.7	0.27 ± 0.2	0.0001
% $CD71^+/TO^+$	22.9 ± 10.9	7.58 ± 4.5	0.002
% Micronuclei			
% MN TO^+	0.77 ± 0.39	8.12 ± 1.9	0.0001
% MN $CD71^+$	0.63 ± 0.22	15.2 ± 3.7	0.0001
% MN $CD71^+/TO^+$	0.14 ± 0.08	1.24 ± 0.97	0.0001
% MN m.e.	0.039 ± 0.023	0.28 ± 0.11	0.0001

Statistical test: Kruskal-Wallis

both TO^+ and $CD71^+$ - in a significant manner (K-W, $p = 0.0001$). In the same way, the ratio of $CD71^+/TO^+$ was reduced, which means that $CD71^+$ reticulocytes were more affected by the treatment. When MN frequencies were analyzed, a clear increase was found in MN in TO^+ cells with HQ treatment (K-W, $p = 0.0001$). The same was observed when only $CD71^+$ cells were evaluated, i.e., MN increased with the HQ treatment (K-W, $p = 0.0001$). Furthermore, a significant increase in MN was also observed in mature erythrocytes with the HQ treatment (Table 4), which means that under the treatment and sampling conditions used in this study, micronucleated cells can reach maturity and can be enumerated for micronucleus induction by a clastogen-like HQ.

The MN frequency in $CD71^+$ cells among the whole population of reticulocytes (i.e. TO^+) was also calculated, and an increase by HQ was also found (K-W, $p = 0.0001$), although the MN frequency taking into consideration only this subpopulation of micronucleated reticulocytes was greatly reduced (Table 4).

It should be noted that the average amount of TO^+ cells analyzed for MN frequency compared to the amount of $CD71^+$ cells analyzed for the results presented in table 4 were significantly different (K-W, $p < 0.05$), as shown in table 5 where raw data are presented.

It can be observed that even though the MN frequency in $CD71^+$ for untreated animals could be calculated by counting 6,000 cells on average, the treatment greatly reduced this number to 1,000; whereas the amount of TO^+ cells was still sufficiently abundant for MN frequency estimation (Table 5). Also, the

total events acquired for these analyses were significantly different between the treatment groups. Still, enough cells could be evaluated to calculate a trustable MN frequency, and the MN frequency calculated either in TO^+ cells or $CD71^+$ cells showed a highly significant correlation (squared R = 0.97, p = 0.00001).

Measurements in humans under the same acquisition conditions

The same approach was used to acquire human samples from healthy children aged 8 to 13 years, and from adults aged 19 to 31 years.

The proportion of TO^+ reticulocytes both in children and adults was similar: 3.9% in children vs 2.4% in adults. The proportion of $CD71^+$ cells, however, was significantly different: 0.1% in children vs 0.05% in adults (K-W, p = 0.0008) (Table 6). Even though the mean ratio $CD71^+/TO^+$ was higher in adults, it was not significantly different from child ratio, which showed less variability.

MN frequencies in TO^+ cells exhibited a difference close to statistical significance (p = 0.08) in adults compared to children (Table 6), whereas the other MN frequencies estimated for reticulocytes, that is to say, percentage MN in $CD71^+$ cells and percentage MN in $CD71^+$ cells/TO^+ cells, showed no significant difference.

Similarly to rat blood, the amount of TO^+ was significantly higher than $CD71^+$ cells (Table 6, Fig. 4b) and even though more events had to be acquired for analyses, enough TO^+ cells could be acquired for both children and adults; however, with three

Table 5. Raw data used to calculate the parameters shown in table 4, per animal.

Cell marker	Cell type[T]	Untreated (mean % ± s.d.)	Hydroquinone (mean % ± s.d.)	p
RNA	TO^+ ret	27,728 ± 778	18,169 ± 7,553	0.04
TfR	$CD71^+$ ret	6,397 ± 3,438	1,158 ± 387	0.06
DNA + RNA	MN TO^+ ret	215 ± 123	1387 ± 410	0.0009
DNA + TfR	MN $CD71^+$ ret	41 ± 27	174 ± 75	0.01
DNA	MN m.e.	153 ± 99	1302 ± 734	0.01
	[#]All events	749,269 ± 83,040	1,057,638 ± 156,993	0.009

Statistical test: Kruskal-Wallis; TfR – transferrin receptor; ret - reticulocyte
[#]: Amount of acquired cells before any gating. [T]: Cells positive to CD71 were also positive to TO.

Table 6. Results obtained from human blood.

% Reticulocytes	Children					Adults					P*
	Mean	s.d.	Median	Min.	Max.	Mean	s.d.	Median	Min.	Max.	
$\%TO^+$	3.9	0.7	3.9	2.8	5.2	2.4	2.0	2.3	0.3	5.5	0.14
$\%CD71^+$	0.1	0.05	0.1	0.02	0.2	0.05	0.03	0.03	0.02	0.16	0.0008
$\%CD71^+/TO^+$	2.4	1.1	2.4	0.8	4.2	4.9	4.5	4.3	0.4	13.0	0.32
% Micronuclei	**Mean**	**s.d.**	**Median**	**Min.**	**Max.**	**Mean**	**s.d.**	**Median**	**Min.**	**Max.**	**P***
% MN TO^+	1.5	0.6	1.3	0.5	3.2	2.5	1.9	2.1	0.6	7.3	0.08
% MN $CD71^+$	6.8	4.3	5.6	2.1	17.7	8.2	5.7	6.7	0	21.3	0.18
% MN $CD71^+/TO^+$	0.12	0.03	0.12	0.08	0.2	0.39	0.43	0.27	0	1.43	0.49
% MN m.e.	0.09	0.05	0.08	0.02	0.2	0.01	0.01	0.007	0.003	0.05	0.0001

*: Comparisons were made between children and adults.

Table 7. Raw data showing the number of cells carrying each cell marker, per individual.

Cell marker	Cell type[T]	Children					Adults					P
		Mean	s.d.	Median	Min.	Max.	Mean	s.d.	Median	Min.	Max.	
RNA	TO^+	23,605	4,867	25,348	13,437	30,101	12,990	10,140	10,427	1,655	31,029	0.003
TfR	$CD71^+$	585	332	541	164	1,210	264	126	251	79	527	0.002
DNA + RNA	MN TO^+	350	189	329	129	810	203	117	176	38	415	0.005
DNA + TfR	MN $CD71^+$	29	8	28	15	51	22	22	16	0	100	0.23
DNA	MN m.e.	505	271	465	166	1182	58	28	46	32	147	0.001
	[#]All events (millions of cells)	1.43	0.34	1.39	0.82	2.28	1.56	0.70	1.37	0.77	3.18	0.93

[#]: Amount of acquired cells before any gating.
Statistical differences refer to comparisons between children and adults.
[T]: Cells positive to CD71 were also positive to TO.

adult samples less than 5,000 TO^+ cells were obtained. With respect to $CD71^+$ cells, they were too scarce in all samples (Table 7), even though the whole stained sample was used during the acquisition step.

Hence, micronucleus frequencies calculated with either cell type were dissimilar, with higher values among $CD71^+$ cells (Table 6), and the correlation between MN in $CD71^+$ and TO^+ cells was greatly reduced (squared $R = 0.1548$, $p = 0.02$) in comparison to the results obtained with rat cells.

A noticeable finding was that adults had on an average half of the amount of TO^+ cells than children; moreover, there were two samples with less than 2,000 TO^+ cells among adults. Likewise, the amount of $CD71^+$ cells was even lower in adults than in children.

Finally, micronucleated cells were more abundant among TO^+ cell fractions than among $CD71^+$ cell fractions (Table 7).

It should be borne in mind that both RNA and the transferrin receptor (CD71) were determined at the same time in every sample, and that all cells carrying the transferrin receptor (CD71) contained RNA, but not the opposite, i.e., not all cells containing RNA carried the transferrin receptor.

DISCUSSION

Our results demonstrate that the proportion of $CD71^+$ reticulocytes in human blood is lower than in rat blood, suggesting that they have a faster rate of maturation in humans than in rats, i.e., the TfR in animals seems to have a larger lifespan in the peripheral blood than in humans, where levels are normally low [23]. Different authors have found varying proportions of $CD71^+$ cells among the human erythrocyte population. Wooley *et al.* [23] reported a mean of 0.4%, whereas Serke and Huhn [24] observed a mean of 0.81% in healthy adults; while evaluating the micronucleus frequency, Dertinger *et al.* [15, 16] have reported a range between 0.04% and 0.51% in healthy subjects, coincident with the levels reported here, and Costa *et al.* [18] reported a mean of 0.04% in adults. Cervantes *et al.* [25] found a wider range between 0.5% and 3.7% in normal children.

Conversely, the amount of RNA containing reticulocytes in humans is considerably higher. According to Wooley *et al.* [23], the transferrin receptor (CD71 or TfR) is lost early in the maturation of circulating reticulocytes, whereas the RNA content detected by TO takes 24-48 hours to be catabolized [26, 27], providing a larger window of opportunity to detect recent genotoxic events. The lifespan of

TfR, if calculated from the frequency observed in our results, is considerably larger in the rat than in humans: 22% of reticulocytes carry the TfR in rats, whereas only 2% to 4% carry it in humans, which would mean that this receptor has a lifespan of 11 hours in rat reticulocytes, whereas it would last only 2 to 4 hours or less in humans (%$CD71^+/TO^+$ in tables 4 and 6).

In the present study, 1.5 x 10^6 events had to be acquired in order to calculate the MN frequency in humans (twice the number of events for untreated rats). However, the amount of $CD71^+$ cells was still very poor as shown in table 7 and the MN frequency calculated for this number of cells was higher than the one calculated for TO^+ cells and more widely variable. It was found that the frequency of MN in $CD71^+$ cells was inversely correlated with the amount of enumerated $CD71^+$ cells (squared R = 0.20, p = 0.003). This variability and inverse correlation was not observed among untreated rats which showed very similar MN frequency with either type of reticulocytes evaluated (Table 4). In order to have a comparable number of evaluable cells carrying the TfR, it is necessary to increase the number of events at least 10X, thus increasing the time of evaluation. This problem has been tried to be circumvented when analyzing human blood, by splitting the samples, generating a low-density subsample to analyze CD71 negative cells and a normal-density subsample to evaluate $CD71^+$ cells and the micronuclei in them [28]. The goal is to count 20,000 $CD71^+$ reticulocytes in the second sample. Nevertheless, in our experience this is not accomplished with normal human samples and very often the sample is totally consumed during the acquisition in the cytometer without reaching not even 2,000 $CD71^+$ cells. This problem has been acknowledged in review papers [29] and more studies were recommended in order to obtain greater reproducibility in human studies among different labs. Our results suggest that it might be wise to reconsider using RNA to identify reticulocytes instead of CD71 antigen of the transferrin receptor to this end.

Experience has shown that the TfR is not the best marker for a rapid, efficient, clean and automated evaluation of micronuclei in human reticulocytes. Reticulocytes, and hence $CD71^+$ cells, have been shown to greatly increase during pathological states such as hemolytic anemia or when intense bleeding has occurred. Under normal conditions, however, the amount of $CD71^+$ cells is consistently low and when individuals are exposed to toxic agents it seems it could be even lower, as has been demonstrated here with the hydroquinone treatment in rats or with antineoplastic treatments in humans [15, 16].

Reticulocytes containing RNA in their cytoplasm are more abundant in human samples, enough to allow for a trustable MN frequency evaluation as has been shown in the present report, where the analysis was restricted to the reticulocytes containing the greatest amount of RNA selected by their fluorescence. This approach was used by Hayashi *et al*. [30] in several studies on the effects of chemicals on the micronucleus frequency of rodent bone marrow and peripheral blood, which led to the adoption of this method as a standard for OECD guidelines, where not only mice but also rats are currently accepted as model animals for the testing of chemicals. These cells retain their ribosomes for 24 to 48 hours, permitting the evaluation of recently induced DNA damage.

Our results with the experiments in rats demonstrated that genotoxicity is detected in TO^+ cells with the same accuracy as it is detected with $CD71^+$ cells. A great correlation was found between the MN frequency in TO^+ cells and those carrying the TfR (squared R = 0.97, p = 0.00001). The same correlation was very low in human samples (squared R = 0.15, p = 0.02), and this could be attributed to the very low amount of $CD71^+$ cells as was demonstrated by the inverse correlation of the MN frequency in these cells with the amount of enumerated $CD71^+$ cells. It was also demonstrated that a large amount of evaluable reticulocytes is ignored when using the TfR antigen CD71 as the biomarker to identify reticulocytes (Fig. 4).

The advantage of using TO^+ cells for MN determination is that RNA has a longer lifespan than the TfR, but not so long as to allow for the elimination of damaged cells by the spleen, as was observed in our results, where more TO^+ micronucleated cells were counted than those carrying the TfR (Table 7). The approach used here to evaluate reticulocyte MN frequency in humans was mainly based on the methodologies recommended by Dertinger *et al*. [16], Grawé *et al*. [5] and Abramsson *et al*. [20] by using a dual laser

cytofluorometer in a blue-violet optical configuration. Many laboratories may not have access to this equipment, but only to a single blue laser. An alternative could be to restrict their determinations to only TO^+ cells, without the identification of $CD71^+$ cells - as we did by using channel BL3 - and, for example, use a new fluorescent dye like DRAQ type instead, which stains DNA in the far red, to identify micronuclei with the same blue laser, but at a very different wavelength (644 nm) from TO (533 nm) in channel BL1, or to use an antibody against DNA conjugated with that same kind of fluorochrome.The advantage of the approach presented here with respect to the methods proposed by Abramsson *et al*. [20] and Offer *et al*. [31] is that no isolation of cells is required, thus greatly reducing the processing time of samples and the amount of blood necessary for the analysis. With the protocol presented here, 10 stained human samples can be acquired in 3.5 to 4 hours.

CONCLUSION

An alternate protocol for the enumeration of micronucleated reticulocytes in microliter quantities of human blood samples was presented based on the identification of RNA containing cells instead of the search of cells carrying the transferrin receptor CD71, which are found in a very low frequency in normal human blood. Only the cells with medium to brighter TO stain were considered for the calculation of MN frequency, which could be done for a robust number of evaluable cells compared to the scarce number of cells carrying the TfR in most human samples. The method is fast, sensitive and requires only one high-density sample for micronucleus determination per individual. Further development can be done to make this method adapt to a cytometer with a single blue (488 nm) laser.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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