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Automated Human Blood Micronucleated Reticulocyte Measurements for Rapid Assessment of Chromosomal Damage

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Abstract

This study evaluated the utility of human blood micronucleated reticulocyte (MN^{CD71+}) frequency measurement as a cytogenetic damage biomarker. The analytical methodology was flow cytometry in conjunction with a previously described three color fluorescence labeling technique that includes anti-CD71 to focus analyses on the most immature fraction of reticulocytes [Dertinger *et al.*, Environ. Molec. Mutagen., 44:427–435 (2004)]. Blood specimens from fifty self-reported healthy adult volunteers were studied. In addition to MN^{CD71+} measurements, blood plasma folate and B_{12} levels were assessed, since these variables tend to influence other indices of cytogenetic damage. Time-course data are also provided for ten cancer patients undergoing treatment. For these subjects, frequency of MN^{CD71+} was measured immediately before therapy, and daily during the first week of chemotherapy and/or fractionated radiotherapy. For the group of healthy volunteers, the variables of age, and folate and B_{12} levels demonstrated no significant effect on MN^{CD71+} frequency. In addition, no difference was observed between pre-treatment MN^{CD71+} values for cancer patients compared with healthy volunteers. Regarding chemotherapy and/or partial body radiotherapy, elevated frequencies were observed upon initiation of treatment for 9 of the 10 patients studied. Maximal effects were observed three to five days following initiation of therapy. The largest increases in frequency of MN^{CD71+} (up to 25.9-fold) were observed in those patients exposed to anti-neoplastic drugs, presumably due to the systemic red marrow exposure provided by these agents. Taken together, these data support the hypothesis that the MN^{CD71+} endpoint represents a valuable biomarker of cytogenetic damage that does not require cell culture or microscopy-based scoring.

Keywords

Micronuclei; cytogenetic damage; DNA damage; flow cytometry; CD71 antigen

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1. INTRODUCTION

In vivo cytogenetic damage assays have proven useful for myriad human health applications, including assessments of product safety, occupational and environmental exposures, dietary and life-style choices, and the effect of genetic polymorphisms on chromosomal integrity (1–6). Furthermore, the current gold standard techniques for estimating radiation dose in the absence of physical dosimetry are based on chromosomal damage endpoints, especially dicentric and micronucleus formation in peripheral blood lymphocytes (7–10). Whatever the application, it is well recognized that conventional assays are too cumbersome to efficiently assess large populations (11–12). Thus, there is a need for new methods capable of quantifying chromosomal damage that are significantly more amenable to application on a mass scale.

Whereas chromosomal effects measured in peripheral blood lymphocytes have traditionally involved cell culture in the presence of mitogens, erythrocyte-based micronucleus assays do not share this requirement. Rather, harvested cells are immediately ready for analysis following minimal processing. As the assay was originally described, target cells (i.e., reticulocytes, or Retics) were obtained from the bone marrow compartment of rodents (13–14). MacGregor and colleagues (15) demonstrated that micronucleated erythrocytes formed in the bone marrow persist in the peripheral circulation of mice, paving the way for mouse blood-based assays. Since the spleen of most other species eliminate micronucleated erythrocytes from circulation, the use of rat, canine, non-human primate, and human blood was considered counterintuitive. Indeed, although splenic filtration does dilute the effect observed in blood relative to the bone marrow compartment, much data have been reported establishing that circulating Retics may represent a suitable target population for studying genotoxicant-induced micronuclei, even for species with efficient splenic activity (16–29). It has been reported that assay sensitivity can be realized for blood-based analyses by restricting interrogation to the most immature fraction of Retics (16,19,21–22,25), and also by increasing the number of Retics evaluated (30). Both of these modifications to the traditional assay are readily made using various flow cytometry-based techniques for measuring the frequency of micronucleated reticulocytes.

The current study was designed to address two deficiencies that exist in the human blood micronucleated reticulocyte literature: sparse information regarding the degree of inter-individual variation, and an incomplete understanding of the sensitivity and kinetics of the endpoint when exposure to known clastogenic agents occurs. To address these remaining questions, a flow cytometry-based assay developed by this laboratory was applied to blood specimens from 50 self-reported healthy volunteers in order to score: (i) young Retics (i.e., CD71-positive erythrocytes, or Retic^{CD71+}), (ii) micronucleus-containing young reticulocytes (MN^{CD71+}), and (iii) micronucleus-containing mature erythrocytes (MN^{CD71-}). As folate and B_{12} levels have been shown to affect other endpoints of cytogenetic damage (31–32), the influence of these factors on MN^{CD71+} incidence was evaluated for the health volunteer population. Finally, flow cytometric analyses were performed for blood specimens obtained from cancer patients before and during chemotherapy and/or partial body radiotherapy.

2. MATERIALS AND METHODS

2.1. Reagents

Prototype Human MicroFlow®^{PLUS} Kits (Litron Laboratories, Rochester, NY) contained anti-human-CD71-FITC, anti-human-CD61-PE, diluent solution, fixative, buffer solution, RNase stock solution, anti-rat-CD71-FITC, propidium iodide solution, and fixed malaria-infected rodent blood (“malaria biostandard”).

2.2. Blood Specimen Acquisition, Processing, and Storage

2.2.1. Healthy Subjects—This study was approved by the University of Rochester Institutional Review Board and the Western Institutional Review Board (WIRB, Olympia, WA); informed consent was acquired from each of the 50 healthy volunteers recruited from the University of Rochester Medical Center. This group comprised twenty-five males and twenty-five females, ranging in age from 21 to 63 years (mean ± std dev = 41 ± 11.4 yrs). Approximately 6 mL of venous blood was collected in green-capped vacutainers (sodium heparin). Approximately 5 mL of blood suspension was pelleted by centrifugation and the plasma was frozen for subsequent measurement of B12 and folate levels , while approximately 1 mL whole blood cell suspension was transferred to tubes containing 5 mL diluent solution. Aliquots (1 mL) of diluted blood were then forcefully injected into 15 mL polypropylene tubes containing 11 mL ultracold fixative solution (−80°C). The tubes were vortexed for several seconds and returned to a −80°C freezer. Fixed samples were stored at −80°C for at least one day before being transported to Litron Laboratories on dry ice. At Litron, samples were stored at −85°C until flow cytometric analysis.

2.2.2. Cancer Patients—This study was approved by the University of Rochester Institutional Review Board and the WIRB; informed consent was acquired from each of the cancer patients recruited from the James P. Wilmot Cancer Center, University of Rochester. As with specimens from healthy volunteers, these samples were collected into heparin vacutainers, diluted, and fixed into ultracold fixative. Blood was obtained just prior to initiation of therapy and again at approximately 24 hour intervals during the first week of treatment. For two patients, blood specimens were collected during the first week of induction chemotherapy without radiation (i.e., cisplatin plus docetaxel) and again four weeks later when concomitant chemotherapy and radiotherapy were initiated. Only those patients able to provide four or more specimens are presented here (n = 10). Subject characteristics are presented inTable 1. Fixed samples were stored at −80°C for at least one day before being transported to Litron Laboratories on dry ice. At Litron, samples were stored at −85°C until flow cytometric analysis. (Cancer patient blood pspecimens were not processed for plasma B_{12} or folate measurements.)

2.3. Measurement of Plasma Folate and B_{12} Levels

Plasma was allowed to thaw at room temperature and 200 μl of each specimen was added to 1 mL freshly prepared Borate-KCN buffer solution with dithiothreitol and ligand-labeled folate, according to instructions included with the Immulite® B_{12} and Folic Acid kits (Diagnostic Products Corp. Los Angeles, California). After a heat denaturation step, levels of B_{12} and folate were measured using the chemiluminescence-based Immulite Instrument (Diagnostic Products Corp.).

2.4. Micronuclei Fluorescence Labeling Technique

Fixed human blood specimens (2 mL) were added to tubes containing 12 mL ice-cold buffer solution and cells were collected by centrifugation. Washed cells were concentrated with vigorous decanting, and entire cell pellets were added to tubes containing 100 μl of an antibody/RNase solution (anti-human-CD71-FITC, anti-human-CD61-PE, RNase A), which was prepared according to MicroFlow kit specifications. Following successive 30 min incubations at 4°C and room temperature, cells were washed with 5 mL buffer containing 1% v/v fetal bovine serum. Finally, cells were resuspended in 1 to 1.5 mL working propidium iodide solution. Stained samples were stored at 4°C or on ice until analysis (same day).

2.5. Flow Cytometry Data Acquisition

At the beginning of each day of flow cytometric analysis, instrumentation and acquisition/analysis software parameters were calibrated based on the fluorescence of a biological standard

—blood from *Plasmodium berghei* infected rats. A 20 μl aliquot of this fixed and washed cell suspension was incubated with 80 μl of antibody/RNase solution, according to manufacturer specifications. These samples guided photomultiplier tube voltage and electronic compensation settings to optimally resolve parasitized Retics (MN^{CD71+} mimicking cells), and also guided the position of the quadrant delineating erythrocytes with and without MN (33–34).

Data acquisition and analysis were performed using a FACSCalibur flow cytometer providing 488 nm excitation, running CellQuest software (v3.3) (instruments and software from BD-Biosciences, San Jose, CA). Anti-CD71-FITC, anti-CD61-PE, and propidium iodide fluorescence signals were detected in the FL1, FL2, and FL3 channels, respectively (log scale). Human blood specimens were labeled and resuspended with propidium iodide solution at high densities. Each specimen was analyzed two times, the first at a reduced cell density (50 to 100 μL high density specimen added to 400 μl ice cold propidium iodide solution). A second flow cytometric analysis was performed on the undiluted, high density sample using an FL1 threshold. Set sufficiently high, this had the effect of eliminating the majority of events (i.e., mature, CD71-negative erythrocytes) from consideration, facilitating rapid evaluation of immature Retics for the presence of micronuclei (35). Figure 1 andTable 2 provide more detail regarding these low and high density analyses.

2.6. Statistical Analyses

The precision of flow cytometic data acquired by the method employed herein, as well as other analytical performance characteristics of the technique, have been described in detail previously (36–37). Statistical analyses were performed with JMP Software (v5, SAS Institute, Cary, NC). For healthy volunteers, the range, mean, and standard deviation for percent $Retic^{CD71+}$, MN^{CD71-}, and MN^{CD71+}, and levels of folate and B_{12} in plasma were calculated. The variables age, and plasma folate and B_{12} levels were evaluated for possible effects on frequency of MN^{CD71+} with the JMP program's regression analyses platform. The associated ANOVA tables partitioned the total variation into components, and compared the linear-fit equation with a simple mean response model. A p value < 0.05 was used to indicate a significant regression effect. Additionally, all cancer patients' pre-treatment MN^{CD71+} frequencies were compared with healthy volunteers' MN^{CD71+} values using a two-tailed, unpaired t-test (significance indicated by $p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Healthy Subjects

Specimens from 50 self-reported healthy adult volunteers were analyzed for $Retic^{CD71+}$, MN^{CD71-}, and MN^{CD71+} frequencies. Fluorescence profiles for these erythrocyte subpopulations are shown in representative bivariate plots (Figure 1), and frequency data are presented inTable 3. Whereas classically defined Retics (i.e., RNA-positive erythrocytes) are typically found in circulation of healthy adults on the order of 1% to 2%, the mean $Retic^{CD71+}$ value was 0.10%. Previous studies with anti-human-CD71 (25–26) have resulted in similar findings, suggesting that it is approximately the youngest 10% to 20% of Retics that label with this immunoglobulin reagent. Presumably, the measurement of micronucleus frequencies in a young age cohort such as this is desirable to help minimize the impact that splenic filtration function has on genotoxicant-induced micronucleus frequencies.

The average frequency of MN^{CD71+} in the plasma of healthy subjects was similar (although somewhat lower) to previously reported values observed in the bone marrow or in the peripheral blood circulation of splenectomized human subjects (0.12% compared with approximately 0.2% to 0.3%, respectively) (3,38–43). This is likely related to splenic filtration function, which

may not be *fully* negated by restricting analyses to Retic^{CD71+} (44). Even so, when compared with MN^{CD71-} values (mean = 0.002%), the average MN^{CD71+} frequency of 0.12% provides evidence that the analytical system described herein does effectively minimize the impact that spleen function has on peripheral blood micronucleus frequency.

Similar to other reports (3,19,41), we observed a considerable range of MN^{CD71+} frequencies in presumably healthy volunteers (0.04% – 0.28%). Linear regression analyses demonstrated that the variation in these baseline readings could not be correlated with age, or levels of B_{12} and folate. This is in contrast to other studies reporting significant effects for each of these factors on the incidence of micronuclei (3,31–32). These discrepancies may be related to differences in target cells (erythroblasts versus lymphocytes) or some other factor(s), such as the relative homogeneity of these subjects, the great majority of which exhibited normal/healthy B_{12} and folate levels (all recruited from a University hospital setting). The design of biomonitoring studies will benefit from further characterizations of inter-individual variation.

3.2. Cancer Patients

As indicated byTable 1, the ten cancer patients provided a cross-section of treatment modalities for evaluating MN^{CD71+} responses. As expected, these therapies tended to reduce the frequency of Retic^{CD71+} within one to two days post-treatment (Figure 2, yy-axis). The proportion of red marrow space that was subjected to treatment was likely an important determinant for the range of responses observed. For instance, those patients that received systemic chemotherapy showed the greatest reduction in frequency of Retic^{CD71+}. In fact, in the case of subjects CP10/12 and CP11/14, cisplatin plus docetaxel treatment caused peripheral blood frequency of Retic^{CD71+} to drop to a level that precluded accurate determination of MN^{CD71+} frequency beyond two or three days post-treatment.

Regarding cancer patients' MN^{CD71+} frequencies, 9 of 10 patients demonstrated elevated levels over the course of therapy (see Figure 2, y-axis). As with Retic^{CD71+}, MN^{CD71+} frequencies are expected to be influenced by the proportion of red marrow space exposed. Thus, in the case of radiotherapy, it is likely that micronucleus induction is muted to the extent that non-exposed sites of erythropoiesis supplied the peripheral blood compartment with MN^{CD71+} at a baseline frequency. This likely explains the nil effect that radiotherapy had on CP17's MN^{CD71+} frequency. This subject exhibited no reduction to %Retic^{CD71+}, suggesting the presence of little or no active red marrow space in the treatment field. Conversely, the higher micronucleus responses observed for patients undergoing chemotherapy can likely be attributed to the large amount of red marrow exposure achieved (e.g., 25.9-fold for CP26). Subject CP21 was an exceptional case in that the frequency of MN^{CD71+} was observed to increase over the week of therapy, even as the frequency of Retic^{CD71+} values increased markedly. It is tempting to speculate that this patient's diagnosis of multiple myeloma, a condition that impacts the red marrow space, may have contributed to this unusual response to treatment.

Despite differences in health status and mean ages, cancer patients did not exhibit a statistically significant difference in pre-treatment MN^{CD71+} frequency relative to the group of 50 self-reported healthy volunteers.

3.3. Conclusions

Data presented herein support the hypothesis that the frequency of MN^{CD71+} in human peripheral blood circulation can be used to index recent chromosomal damage induced by agents and dose levels/intensities used in the cancer clinic. This is made possible by a high throughput analytical system capable of restricting analyses to the most immature fraction of Retics. The rarity of Retics^{CD71+}, coupled with the low rate of micronucleus formation, makes high analysis rates an essential characteristic. These data, coupled with recent reports (27–

29), suggest that Retics^{CD71+} may represent a viable alternative to lymphocyte-based analyses. Retics offer several advantages, including a low blood volume requirement, no need for cell culture, and compatibility with an automated scoring methodology. Of course, when exposures occur acutely, the short-lived nature of genotoxicant-induced MN^{CD71+} relative to more persistent expression of chromosome damage in lymphocytes may favor analysis of one cell type over the other, depending on the experimental question being asked. Since MN^{CD71+} induction was evident in clinical specimens originating from the cancer clinic, it will be important to assess the utility of the endpoint for studying the genotoxic consequences of occupational, environmental, nutritional, and/or genetic factors. Use of this method for biomonitoring applications such as these is less certain, but reports by Grawé *et al.* (28) and Stopper *et al.* (29) are encouraging.

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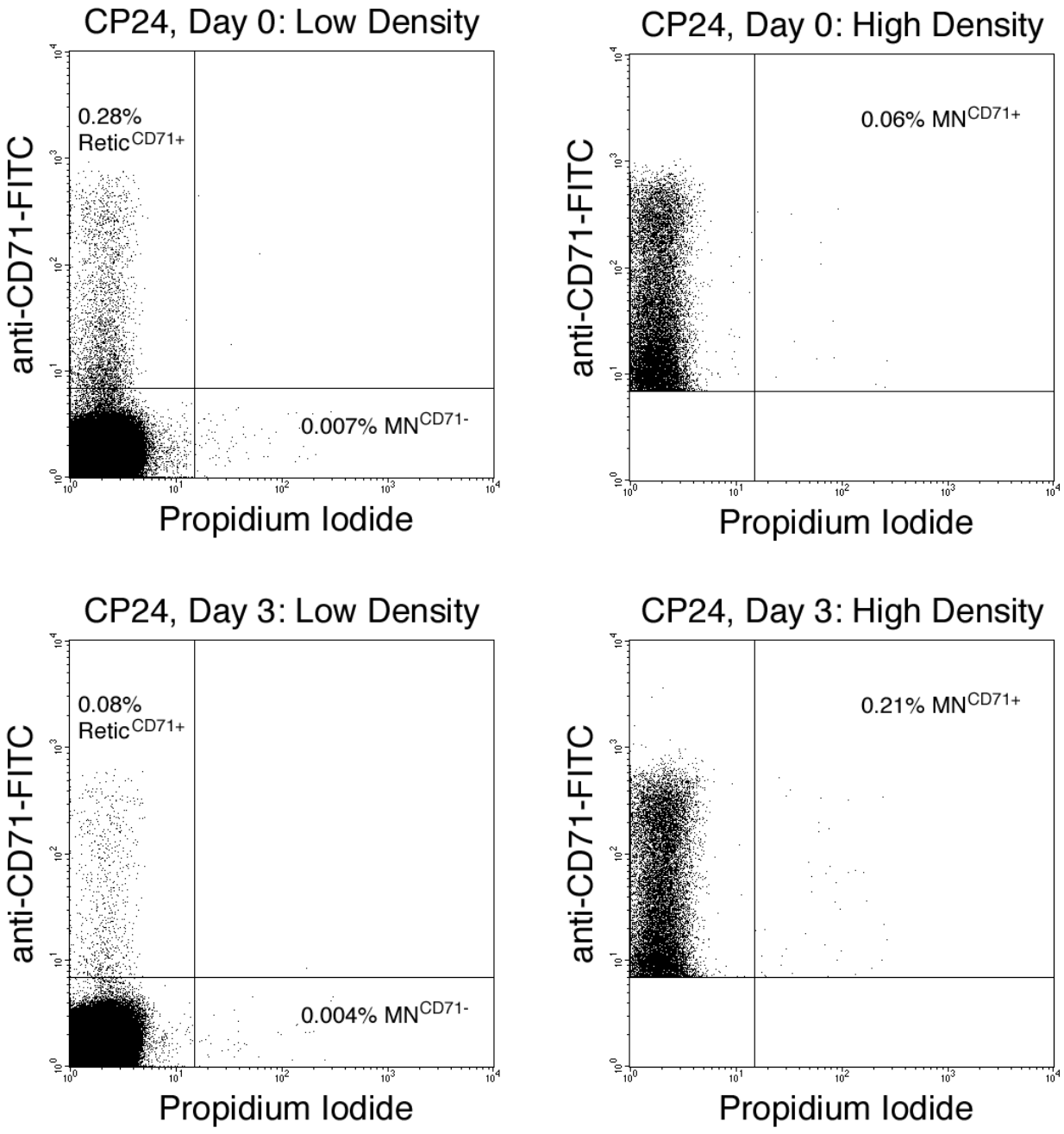

Figure 1.

Bivariate graphs illustrate the fluorescent resolution of human erythrocyte subpopulations (nucleated cells and platelets have been excluded from these plots based on their light scatter and fluorescence staining characteristics). Upper left and upper right plots: low and high density analyses of Cancer Patient CP24. Lower left and lower right plots: low and high density analyses of Cancer Patient CP24, three days following initiation of treatment. The low density analyses occurred with a forward scatter threshold, facilitating enumeration of young reticulocytes ($Retic^{CD71+}$), and also CD71-negative micronucleated erythrocytes (MN^{CD71-}). The high density analyses occurred with FL1-thresholding, permitting rapid measurement of CD71-positive micronucleated reticulocytes (MN^{CD71+}).

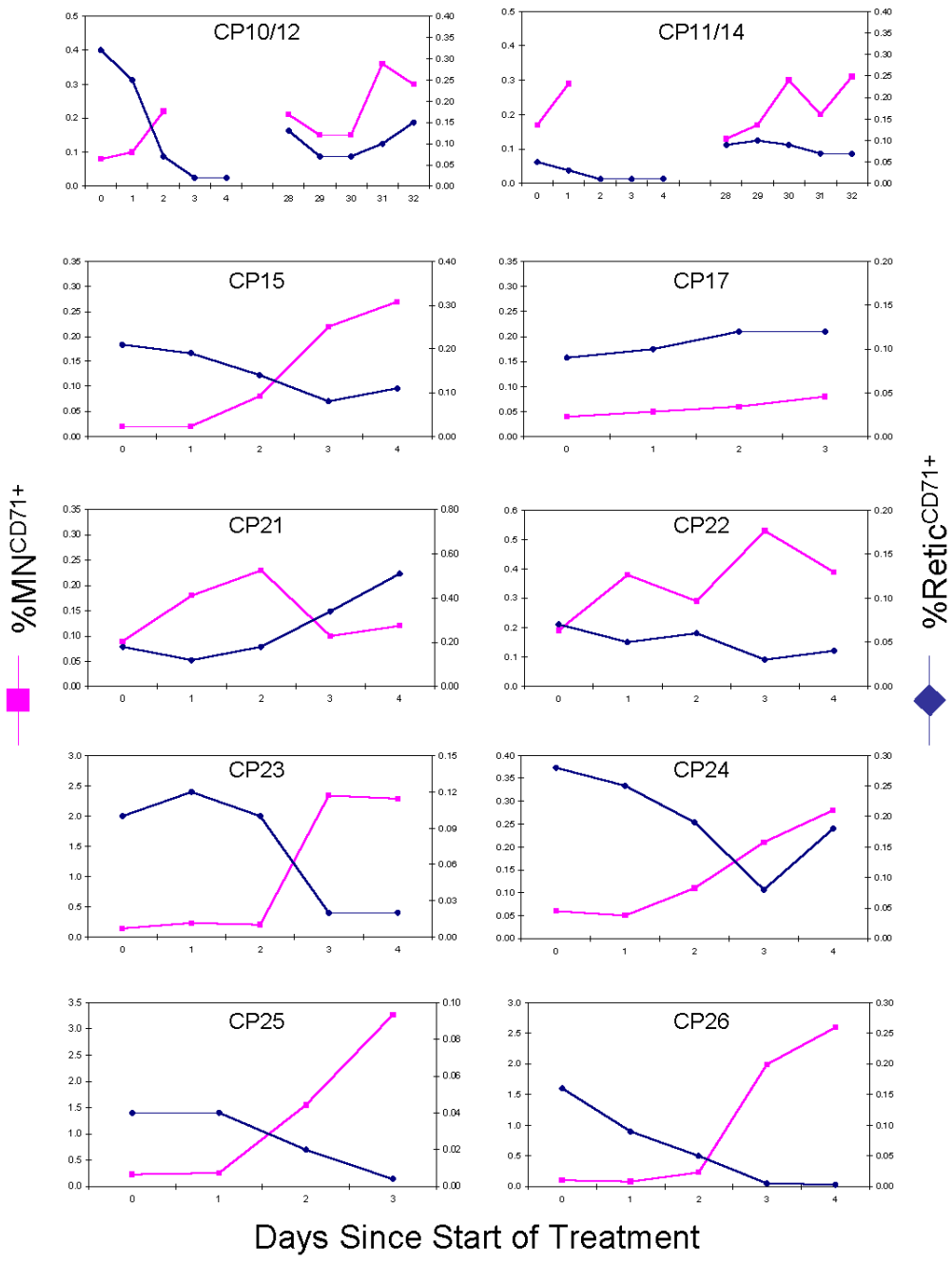

Figure 2.

Percent CD71-positive micronucleated reticulocytes (MN^{CD71+}) and percent CD71-positive reticulocytes ($Retic^{CD71+}$) are graphed for each cancer therapy patient. Treatment details are shown inTable 1. While the frequency of $Retic^{CD71+}$ was generally found to decline over the first week of treatment, higher incidences of MN^{CD71+} were observed.

Table I

Cancer Patient Information.

ID	Sex	Age	Diagnosis	Treatment	Radiation Dose (Gy/day)
CP10/12	M	75	NSCL Cancer IIIB	1st Rx = cisplatin (75 mg/m^2) plus docetaxel (75 mg/m^2)	N/A
				2nd Rx = RT Mon. – Fri. (chest) plus Mon. & Thurs. docetaxel (12 mg/m)	1.8
CP11/14	M	56	NSCL Cancer IIIB	1st Rx = cisplatin (75 mg/m^2) plus docetaxel (75 mg/m^2)	N/A
				2nd Rx = RT Mon. – Fri. (chest) plus Mon. & Thurs. docetaxel (12 mg/m)	1.8
CP15	M	75	Mesothelioma	RT Mon. – Fri. (chest) plus Mon. & Wed. & Fri. paclitaxel (20 mg/m^2)	1.8
CP17	M	64	NSCL Cancer, UC	RT Mon. – Fri. (chest)	2.5
CP21	M	71	Multiple Myeloma	RT Mon. – Fri. (right and left femur, partial pelvis)	3.0
CP22	F	46	NSCL Cancer IIIA	RT Mon. – Fri. (chest) plus Mon. & Wed. & Fri. paclitaxel (20 mg/m^2)	1.8
CP23	F	52	NSCL Cancer IIIA	RT Mon. – Fri. (chest) plus Mon. cisplatin (25 mg/m^2)	1.8
CP24	M	52	NSCL Cancer IIIB	RT Mon. – Fri. (chest) plus Tues. docetaxel (20 mg/m^2)	1.8
CP25	F	61	SCL Cancer	Tues. carboplatin (AUC 5) plus Tues. – Thurs. etoposide (100 mg/m^2)	N/A
CP26	M	76	SCL Cancer	Mon. carboplatin (AUC 5) plus Mon. – Wed. etoposide (75 mg/m^2)	N/A

Abbreviations: RT = radiotherapy (megavoltage external-beam photon radiation delivered with linear accelerators); UC = Undifferentiated Carcinoma; NSCL = non-small cell lung; SCL = small cell lung; AUC = area under the curve.

Table II

Flow Cytometric Measurements.

Analysis	Relative Cell Density	Threshold	Stop Mode	Statistic Measured	Endpoint
1	Low	FSC	10^6 RBCs	%Retic $^{CD71+}$ = CD71+ reticulocytes among total RBCs %MN $^{CD71-}$ = micronucleated CD71- RBCs among total CD71- RBCs	Erythropoiesis function Erythrophagocytic activity, primarily splenic
2	High	FL1	20,000 Retic^{CD71+}	%MN $^{CD71+}$ = micronucleated CD71+ reticulocytes among total CD71+ reticulocytes	Genotoxicity (recent)

* Abbreviations: FSC = forward light scatter; RBCs = red blood cells; FL1 = fluorescence channel 1 (corresponds to anti-CD71-FITC fluorescence).

Table III

Healthy Adult Subjects, n = 50

Parameter	Min.	Max.	Avg.	Std. Dev.	P value*
%Retic^{CD71+}	0.02	0.55	0.10	0.093	
%MN^{CD71+}	0.04	0.28	0.12	0.062	
%MN^{CD71-}	0.001	0.006	0.002	0.001	
Age (years)	21	63	41	11.4	0.3721
B_{12} (pg/ml)	99	990	392	205	0.4059
Folate (ng/ml)	6.4	57.1	22.6	11.0	0.6735

*Linear regression analyses evaluating the factors Age, B_{12}, and Folate on MN^{CD71+} frequency; these P values (> 0.05) indicate that these factors do not affect %MN^{CD71+}.