Preliminary Evaluation of Rat Bone Marrow Samples Using the Sysmex XT-2000*i*V

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Accurate and precise examination and classification of animal bone marrow is frequently required for novel preclinical drug evaluation. At present, the techniques employed are often imprecise and labour intensive, requiring highly skilled staff.
We previously evaluated the Automated Hematology Analyzer XT-2000iV (Sysmex XT-2000iV) for peripheral blood haematology parameters in three laboratory species[1,2]. In this study, we examined the use of this haematology analyser to evaluate rat bone marrow suspensions from rat femurs, with comparison to Cytospin™ preparations and cell counts obtained using flow cytometry.
The Sysmex XT-2000iV was able to perform a total nucleated cell count, cell differential counts and myeloid:erythroid ratios for rat bone marrow suspensions within 45 secs of aspiration. Total nucleated cell counts using the Sysmex XT-2000iV showed good correlation with counts obtained using the Siemens Advia 120™.

Key Words Bone Marrow, Rat Bone Marrow, Sysmex XT-2000*i*V.

INTRODUCTION

The rationales and methodologies used for animal bone marrow cell examinations in preclinical animal toxicological studies have been expertly reviewed elsewhere [3-5]. These examinations are important where there is evidence of haemotoxicity from blood cell counts e.g., with anticancer compounds and immunosuppressive agents. Differential counting of bone marrow cells is a complex task due to the variety of cells representing multiple lineages at different stages of maturation. Microscopic examination of bone marrow aspirates and smears requires highly skilled personnel and, ultimately, depends on quality of the bone marrow preparation and the expertise of the analyst. The counts are imprecise due to the small number of cells counted, and they are time consuming and labour intensive. Often the results obtained by this technique are summarised by expressing the relative proportions of granulocytic and erythrocytic cells as the myeloid: erythroid ratio (M:E ratio), with additional comments on cell morphology.
A number of flow cytometric techniques, for examining animal bone marrows with a variety of fluorescent antibody dyes, have been described as alternatives to manual counting techniques [6-10]. The automated counting of rat bone marrow cell differentials improves the counting precision as many more cells are counted and this increases reproducibility. Problems associated with these flow cytometric methods have included the failure to count nucleated red blood cells, difficulties in correctly differentiating various immature cells and the interference of fat particles, microfibres and cell aggregates of variable size [11]. Adipocytes, which are a major component of the bone marrow, can cause erroneous cytograms where the nuclei of fat cells are picnotic and smaller than those of erythroblasts [12]. Using automated haematology analysers for the quantitative analysis of bone marrow previously has been difficult due to several technical problems including the preset cell gatings, although several of these analysers have been used for total nucleated cells counts.
In this study, we evaluated the potential use of an automated haematology analyser to measure rat bone marrow cell subpopulations, produce a total nucleated count, differential cell counts and subsequently to derive M:E ratios.
The Automated Hematology Analyzer XT-2000*i*V (Sysmex Corporation; Kobe, Sysmex XT-2000*i*V) has been developed to perform total and differential leucocyte counts in two different channels by flow cytometry using a semi conductor laser [13]. The analyser is equipped with a manual gating system which allows the

operator to establish individual gates for cell subpopulations. In previous studies of whole blood samples, we have found the Sysmex XT-2000*i*V to be easy to use and reliable with no breakdowns or software problems occurring during previous evaluations [1]. Several publications have reported the use of the Sysmex XT-2000 for human bone marrow studies [14-16].
Data for imprecision, carry-over between samples, linearity and stability were obtained using rat bone marrow suspensions and the Sysmex XT-2000*i*V, and these results were compared to those obtained with a flow cytometric method.

MATERIALS AND METHOD

Bone Marrow Sampling

At necropsy, following euthanasia under isofluorane inhalation and exsanguination, the left femurs were taken from control animals in various studies: the Wistar rats were aged approximately 10 weeks. For consistency the left femurs were used, although no significant differences between left and right femurs have been reported [8,9], The femurs were put into labelled 12 × 75mm polypropylene tubes and placed on ice before further processing.
Equal volumes of phosphate buffered saline (0.1M pH7.4) (PBS) and heat- inactivated foetal bovine serum (FBS) were mixed together and stored at 4°C or on ice (PBS + FBS): this solution was freshly prepared. A second reagent (PBS + BSA) was prepared by adding 8.4mL of 30% bovine serum albumin (BSA) to 500mL 0.1M pH7.4 PBS. This solution was stored at 4°C and was stable for one month.
Attached tissues were removed from each femur using a scalpel, and the ball of the hip joint and the knee of the femur are cut away with bone snips leaving as much of the bone shaft intact. A hole was created in the knee end using a 20-gauge needle held on a 3mL syringe containing 2-3mL of the PBS + FBS mixture. The needle was then pushed into the hip end and the PBS + FBS solution was flushed through and into another 12 × 75mm polypropylene tube. The PBS + FBS solution was drawn back through the bone and expelled several times to flush out the bone marrow. This procedure was carried out within 15 minutes of euthanasia.
The resulting marrow suspension was thoroughly mixed and filtered through a 10µm nylon mesh filter into a clean 12 × 75mm polypropylene tube, and then stored on ice before the washing stage. The filtered bone marrow suspension was then carefully layered onto 1mL of the FBS reagent in a plastic tube and centrifuged at 300g for 5 minutes at 4°C. The supernatant was discarded and the remaining pellet re-suspended in 1mL of the PBS + BSA reagent.
These marrow suspensions were then analysed by Cytospin™ microscopy (for differential counts), using a flow cytometer (for M:E ratios), and the Sysmex XT-2000*i*V. The following cell populations were counted ~ total nucleated cells, eosinophils, neutrophils, immature granulocytes, lymphocyte and monocytes combined, blasts, nucleated red cells, and the percentages of nucleated erythroid, myeloid and lymphoid cells.
In addition, total nucleated cell counts (TNC) were measured in bone marrow suspensions (n = 100) using the XT-2000*i*V and Advia 120™ (Siemens Diagnostics, Frimley UK), within 3h of collection. Other cell populations could not be compared as the Siemens Advia 120™ did not have a suitable gating system for these studies.

Cytospin™ Preparation

To prepare a readable Cytospin™ preparation, the optimum number of cells required is 10^5 cells, i.e.using 100µL of bone marrow cell suspension with a cell concentration of 1×10^6 cells/mL. The total nucleated cell count obtained from the Sysmex XT-2000*i*V is reported in units of 10^9cells/L i.e.,10^6 cells/mL. The following formula has been produced to calculate the amounts of PBS + BSA required to dilute the bone marrow cell suspension to obtain 1×10^6 cells/mL.

$V1 \times C1 = V2 \times C2$
where:
V1 = 100µL of bone marrow suspension
C1 = Total Nucleated count (TNC) from Sysmex XT-2000*i*V
V2 = amount of PBS + BSA to be added
C2 = 1×10^6 cells/mL

100µL of this suspension was used to prepare cells onto a labelled glass slide using a Shandon Cytospin II centrifuge spun at 800g for 5 minutes. After air drying the slide was stained using a Hematek™ 2000 flatbed staining machine using a modified Wright's stain. A 400-cell differential was performed microscopically for each Cytospin™ preparation, with cells classified as erythroid, blasts, immature granulocytes (promyelocytes and myelocytes) and mature granulocytes (bands and segmented).

Flow Cytometry

The flow cytometry staining procedure used the fluorochrome [2,7]-dichlorofluorescein-diacetate (DCF), [8]. DCF (0.2mM) was prepared from a stock 20mM solution diluted with 0.1M PBS immediately prior to use. 100µL of 0.2mM DCF was added to 500µL of bone marrow suspension, and incubated for 15min in the dark at room temperature. 5µL of 6.5mM hydrogen peroxide was then added to decrease background staining, and this mixture was incubated for a further 15min in the dark at room temperature. A negative control without DCF was also included. A Beckman Coulter Epics™ XL flow cytometer was used to count 35,000 events in these samples. The DCF absorbance/emission wavelengths were 495/529nm, and fluorescence was detected using the FL1 channel.

Sysmex XT-2000*i*V

The Sysmex XT-2000*i*V allows the gating of cell subpopulations, and these gate settings can be provided by the manufacturer (Sysmex Corporation, Kobe, Japan) or defined by the individual user. These user definable

gates can be stored and used to analyse subsequent samples, and to re-analyse data for previous samples on the database. Initial comparison studies using the cell gate settings for rat bone marrow samples supplied by Sysmex UK were performed using the DCF flow cytometry method and samples prepared using a Cytospin™ and stained with a modified Wright's stain. On the basis of these comparisons, the initial gate settings were modified to exclude fat particles and improve the gatings of cell populations: these modified settings were used to obtain the data presented here. One set of gatings was produced to obtain cell differentials (later referred to as the Rat Marrow Protocol), and one set was produced to obtain M:E ratios (later referred to as the Flow Protocol). An example of a bone marrow suspension analysed with these settings is shown in ***Fig. 1***.

Imprecision and Linearity

Within batch imprecision was determined by replicate sampling of prepared rat bone marrow samples (n = 10). Linearity was determined by centrifuging a prepared rat bone marrow sample at 400g for 10 minutes. Most of the resulting supernatant was then removed and the remaining sample mixed thoroughly to produce a sample with the highest values (designated the 100% sample). This 100% sample was then subsequently diluted with the removed supernatant to obtain samples with values ranging from 5 to 90% and analysed using the Rat Marrow Protocol and Flow Protocol on the Sysmex XT-2000*i*V.

Carry-over between samples

This was assessed using the recommendations of the I.C.S.H. [17]. Triplicate assays of a sample with high values were followed by triplicate assays of a sample with much lower values using the Rat Marrow Protocol on the Sysmex XT-2000*i*V. The percentage of carry-over for the bone marrow parameters was calculated from the formula given in the guidelines.

Sample Stability

Rat bone marrow suspensions (n = 10) were analysed within 3h of collection (0h), and then stored at 4°C before subsequent analyses at 5 and 24h. Another set of bone marrow suspensions were divided into 2 sets (n = 35): the first set was stored at 4°C, and the second set were stored at room temperature (RT). These two sets of samples were then re-analysed after storage for 72 hours. All samples were brought to room temperature before analysis using the Rat Marrow Protocol and Flow Protocol on the Sysmex XT-2000*i*V. The percentage differences between the first analysis and the subsequent analyses were calculated.

Comparion between rat bone marrow samples using the XT-2000*i*V and Advia 120™

Rat bone marrow samples were analysed within 3h of collection using both analysers for Total nucleated count (n = 100).

Comparion between rat bone marrow Cytospin™ and Flow cytometry suspensions and results from the XT-2000*i*V

Rat bone marrow samples were prepared as described and analysed using the Sysmex XT-2000*i*V and compared with Cytospintm preparations, read microscopically (n = 15) and flow cytometry samples analysed using EPICS™ flow cytometer (n = 38).

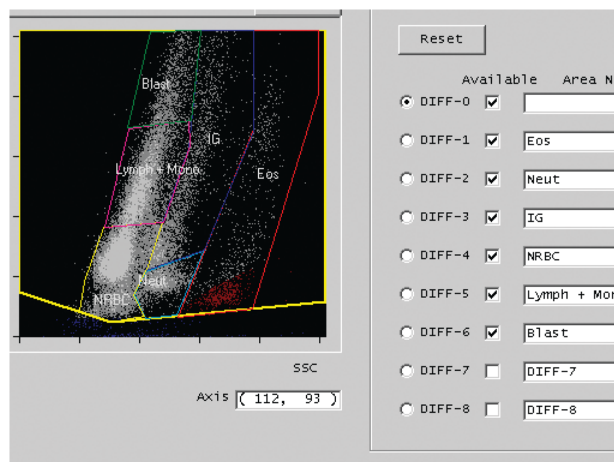

Fig. 1 *Rat Protocol gating using the Sysmex XT-2000iV*

RESULTS

Within-Batch imprecision data for the bone marrow parameters are shown in ***Table 1*** for both rat marrow suspension differentials and M:E ratios using the Sysmex XT-2000*i*V. Acceptable linearity was observed over the ranges obtained, with correlation coefficient values (R^2) of > 0.9844 (***Table 2***). The data for carry-over between samples for the cell populations are shown in ***Table 3***. The counts for bone marrow suspensions proved to be relatively stable when stored at 4°C for 24h: cell numbers decreased in suspensions stored at room temperature (***Tables 4 and 5***).

The data obtained for total nucleated counts using the Sysmex XT-2000*i*V and Siemens Advia 120™ showed good agreement with a correlation coefficient of 0.9821 over the range (***Table 6***). The mean values of the total nucleated cell counts (× 10^9/L) were 45.86 (Range = 5.61 - 95.04 × 10^9/L) using the XT-2000*i*V, and 46.70 (Range = 5.04 - 100.29 × 10^9/L) using the Advia 120™.

Table 1 *Within Batch imprecision data for rat bone marrow (n = 10) using the XT-2000iV*

	Mean	Standard Deviation	Coefficient of variation (%)
Rat Marrow Protocol			
Cell counts (× 10^9/L)			
Total Nucleated	71.52	0.71	0.99
Eosinophils	1.67	0.05	2.97
Neutrophils	4.31	0.17	3.96
Immature Granulocytes	6.79	0.21	3.17
Nucleated Erythroid	25.77	0.61	2.35
Lymphocytes & Monocytes	15.79	0.24	1.54
Blasts	2.31	0.12	5.23
Flow Protocol			
Percentage of cells			
Nucleated Erythroid	47.5	1.00	2.11
Nucleated Myeloid	33.7	0.75	2.24
Nucleated Lymphoid	19.5	0.34	1.76
Myeloid: Erythroid Ratio	0.71	0.03	4.38

Table 2 *Linearity correlation coefficient values obtained for rat bone marrow samples with the XT-2000iV*

	Range	Correlation Coefficient (R^2)
Rat Marrow Protocol		
Cell counts (× 10^9/L)		
Total Nucleated	0.54 - 225.76	0.9844
Eosinophils	0.01 - 6.60	0.9869
Neutrophils	0.06 - 30.15	0.9961
Immature Granulocytes	0.06 - 31.60	0.9890
Nucleated Erythroid	0.24 - 121.16	0.9962
Flow Protocol		
Total Nucleated cells (10^9/L)		
Percentage of cells	0.52 - 221.36	0.9845
Nucleated Erythroid (%)	0.24 - 118.80	0.9927
Nucleated Myeloid (%)	0.19 - 95.02	0.9944

Table 3 *Mean values and percentage carry over between low and high triplicate measurements using the XT-2000iV for rat bone marrow samples.*

Cell counts ($\times 10^9$/L)	Low analyte	High analyte	Low analyte	Carry Over (%)
Total Nucleated Cells	29.73	95.01	25.45	6.15
Eosinophils	0.77	2.40	0.58	10.44
Neutrophils	1.63	7.15	1.51	2.13
Immature Granulocytes	3.24	9.49	3.00	3.70
Nucleated Red Cells	10.53	34.22	8.78	6.88
Lymphocytes & Monocytes	7.02	17.10	5.91	9.92
Blasts	1.15	2.67	1.12	1.94

Table 4 *Mean percentage differences between values obtained within 3h of collection, at 5 and 24h post collection and storage at 4℃ for rat bone marrow samples (n = 10)*

	Mean percentage differences of measurement value	Mean percentage differences of measurement value
	5 - 0 h	24 - 0 h
Rat Marrow Protocol		
Total Nucleated cells	-2.98	-5.47
Eosinophils	0.1	0.0
Neutrophils	0.5	-0.9
Immature Granulocytes	0.1	-1.5
Nucleated red cells	-0.8	1.4
Lymphocytes & Monocytes	-0.2	0.3
Blasts	0.2	0.6
Flow Protocol		
Nucleated Erythroid Cells	-0.3	0.9
Nucleated Myeloid Cells	0.0	-0.9
Nucleated Lymphoid Cells	-0.1	0.2
Myeloid: Erythroid Ratio	0.00	-0.03

Table 5 *Mean percentage differences between values obtained within 3h of collection, and following storage at 4℃ or Room Temperature (RT) for 72h for rat bone marrow samples (n = 35).*

	Mean percentage differences	
	4°C - Fresh sample	RT - Fresh sample
Rat Marrow Protocol		
Total Nucleated cells	7.48	37.71
Eosinophils	0.00	14.84
Neutrophils	-23.14	-26.68
Immature Granulocytes	4.08	59.91
Nucleated red cells	-0.35	21.27
Lymphocytes & Monocytes	36.68	92.01
Blasts	46.83	94.63
Flow Protocol		
Nucleated Erythroid Cells	-4.14	-2.53
Nucleated Myeloid Cells	-12.89	-34.85
Nucleated Lymphoid Cells	42.41	91.46
Myeloid: Erythroid Ratio	-8.38	-34.13

Table 6 *Comparison of rat bone marrow total uncleated cell counts obtained using the XT-2000iV and Advia 120 ™ (n = 100) (R^2 = 0.982), cell differential counts obtained by a manual Cytospin ™ counting procedure (n = 15) and flow cytometry (n = 38)*

Cell population	Mean		Coefficient of variation (%)	
	XT-2000*i*V	Advia 120™	XT-2000*i*V	Advia 120™
Rat Marrow Protocol				
Cell counts (× 10^9/L)				
Total Nucleated cell count	45.86	46.70	50.84	54.26
		Cytospin™ method		Cytospin™ method
Mature granulocytes	155	15	15.54	27.31
Immature Granulocytes	17	25	11.10	15.53
Nucleated red cell count	61	51	6.93	7.76
Blasts	7	9	13.27	18.00
Flow Protocol				
Percentage of cells		Flow cytometry method		Flow cytometry method
Nucleated Erythroid	46.8	48.0	7.48	10.82
Nucleated Myeloid	36.8	34.0	10.16	14.35
M: E Ratio	0.80	0.72	17.89	22.68

DISCUSSION

The imprecision data were considered acceptable and comparable with published literature [15,18]. Similarly, linearity and carry-over between sample data were acceptable. Predictably the data for samples stored at room temperature demonstrated the lability of bone marrow cells: bone marrow cell analysis should be performed as rapidly as possible after collection.

The comparison of total nucleated cell counts obtained using the Sysmex XT-2000*i*V and Siemens Advia 120™ showed excellent correlation and these data were comparable with other published data using the XT-2000*i*V [18]. The cell differential counts obtained by the Sysmex XT-2000*i*V compared well with the Cytospin™ method with lower coefficients of variation using the automated method. The percentage mean values for the nucleated erythroid and myeloid cells were similar, but the small differences had an apparently marked effect on the mean myeloid: erythroid ratios.

For laboratories without access to dedicated flow cytometers, the Sysmex XT-2000*i*V provides an alternative dual function analyser for both blood samples and rapid determination of bone marrow myeloid:erythroid ratios. We were also able to produce a satisfactory gating protocol to count cell subpopulations which compared well with the labour intensive Cytospin™ method. The small sample volume required i.e., 85µL allows other further investigations. Separation methods using specific antibodies, [19] should be possible to adapt the Sysmex XT-2000*i*V technology to obtain further information on bone marrow cells.

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