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Cytological Examination and Cellular Composition of Bone Marrow in Healthy, Adult, Cynomolgus Monkeys (*Macaca fascicularis*)

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Abstract. The purpose of this study was to evaluate the cellular composition of the bone marrow of cynomolgus monkeys (*Macaca fascicularis*). Femoral bone marrow smears from 23 healthy, adult animals (11 males and 12 females) were examined. For each animal, three femoral bone marrow smears were prepared immediately after euthanasia and stained with May–Grünwald–Giemsa. On two of the three smears available, and for each of these smears, a 500-cell differential count was performed and the myeloid: erythroid (M:E) ratio established. The M:E ratio for males varied from 0.67:1.00 to 1.85:1.00 with a mean of 1.03:1.00 and for females from 0.67:1.00 to 1.63:1.00 with a mean of 1.02:1.00. The mean percentage of granulocytic, lymphocytic, plasmacytic and erythroid series was 47.60, 5.44, 1.45 and 46.05% for males and 47.28, 5.12, 1.49 and 46.28% for females. No significant differences were noted between males and females. All cell lines were well represented and showed normal maturation in both sexes. Megakaryocytes were adequate in number and morphology in all animals. Cynomolgus monkeys showed a bone marrow composition similar to rhesus monkeys (*Macaca mulatta*). Cytological examination of bone marrow was found to be a simple and rapid procedure, well suited to the toxicological research environment. It provided excellent information on cell distribution, morphology and maturation of the haematopoietic system.

Keywords: Bone marrow; Cynomolgus monkey; Cytologic examination; *Macaca fascicularis*

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Introduction

Morphological evaluation of bone marrow is an integral part of the assessment of the haematopoietic system and is mostly indicated when abnormalities are observed in the peripheral blood, suggesting a pathological condition of the bone marrow (Jain 1986). In toxicological research, bone marrow examination is one of the numerous parameters assessed to determine the potential toxicity of a compound (Irons 1991). The cynomolgus monkey (*Macaca fascicularis*) has become increasingly used as an experimental animal, but to date there has been no definitive report on the normal cytological composition of the bone marrow in this animal. The purpose of our study was to evaluate the bone marrow cytological composition of healthy adult cynomolgus monkeys, to establish the myelogram of both males and females, and to determine if a sex-related difference existed. Further, we wanted to compare these data with the data published for the rhesus monkey (*Macaca mulatta*).

Material and Methods

Femoral bone marrow smears were retrospectively selected from monkeys that had been used as control animals in oral toxicity studies.

Animals

A total of 23 healthy, young adult, sexually mature, cynomolgus monkeys (11 males and 12 females) were selected from the control groups of oral toxicity studies

performed in an independant research laboratory. These animals had been purchased from a commercial laboratory animal supplier (Charles River/BRF, Houston, TX, USA).

Environmental Conditions

Following acclimatisation and quarantine, the monkeys were selected and randomised into control and treated groups. Animals were housed individually in stainless steel cages, each equipped with a bar-type floor and automatic watering valve. Environmental conditions were monitored and controlled. Standard certified commercial primate food (Purina Primate Chow No.5048, Purina, MO, USA), analysed by the manufacturer for contaminants and municipal tap water, softened, purified and sterilised were offered ad libitum. Monkeys were cared for according to the AAALAC (American Association for the Accreditation of Laboratory Animal Care) (ILAR 1984) and the (Canadian Council on Animal Care) (CCAC 1993) guidelines. Study protocols were approved by the research's institution Animal and Care Use Committee where the studies were performed. Control group animals received daily oral administration of distilled water or methycellulose (Sigma Cheminal Co., MO, USA), depending on which vehicle was used for the compound investigated.

Laboratory Investigations

On the day prior to the termination of each oral toxicity study, blood samples for laboratoy investigations (complete blood count (CBC) and serum chemistry profile) were collected from all monkeys. Blood was drawn from the femoral vein of fasted animals. On the same occasion, urine samples were collected for urinalysis, following an overnight water deprivation.

Haematological parameters were measured in EDTA (ethylenediamine tetraacetic acid) anticoagulated blood using a Coulter' S Plus IV (Coulter Co., Hialeah, FI, USA) and included: haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), red blood cell count, total and differential white blood cell count and platelet count. Erythrocyte and leucocyte morphology were evaluated by light microscopy on modified-Wright stained blood films.

Serum chemistry parameters were measured on a Hitachi 717 automated analyser (Hitachi, BMC Canada, Laval, Quebec, Canada) using the manufacturer's reagents and methods. Parameters studied were: blood urea nitrogen (BUN), creatinine, total protein, albumin, globulin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, glucose, cholesterol, triglycerides, sodium, potassium and calcium.

Urine samples were analysed with commercial reagent strips (Ames Reagent Strips, Ames, IA, USA) and included pH, glucose, ketones, urobilinogen, blood, protein, bilirubin and nitrite. Centrifuged urine sediments were examined by light microscopy.

Bone Marrow Smear Preparation and Examination

At the completion of each oral toxicity study, animals were euthanised and a complete necropsy was performed on each monkey. Immediately following euthanasia, the femur was exposed by incising through skin and muscles of the caudal and lateral aspects of the rear limb. Once exposed, the femoral bone was cut at its proximal aspect. Using forceps, a small plug of marrow was gently extracted from the sectioned bone. A drop of cynomolgus monkey serum, previously mixed with a small quantity of EDTA, was placed on one extremity of a glass slide. The marrow was gently mixed with the serum+EDTA and the material spread in an even film on a glass slide and air dried. Three smears were prepared for each animal.

Bone marrow smears were stained with May-Grünwald-Giemsa according to a standard manual procedure (Lynch et al. 1969). Two of the three femoral bone marrow smears prepared for each animal were stained and examined. For each bone marrow smear, using light microscopy, the same cytologic examination procedure was followed, modified from a previously described method (Lewis and Rebar 1979, Harvey 1984). In a first step, using a low magnification objective (4X–10X), the bone marrow smear was scanned to assess cellularity (i.e. number of cells on the slide) and quality (i.e. presence of bone marrow particles/spicules). The overall marrow cellularity was not evaulated as this is more accurately achieved through bone marrow histological examination (Harvey 1984). Additionally, the megakaryocytic series elements, mostly megakaryocytes, were evaluated with regards to number, morphology and maturation. In a second step, using high magnification objectives (50X oil–100X oil), a 500 cell differential count was performed. Differential count included cells from the erythroid, granulocytic, lymphocytic and plasmacytic series. Morphology and maturation sequence of each cell line were assessed. Non-haematopoietic elements were observed but not counted. The myeloid to erythroid ratio (M:E) was calculated and the percentages of each cell line established.

Calculations and Statistics

Numerical data (i.e. M:E ratio and percentages) derived from the bone marrow smear examination were subjected to the calculation of group mean values and standard deviations for each sex and were statistically compared for sex differences by analysis of variance test (Anova Procedure, SAS System, SAS, NC, USA).

Results

Laboratory Investigations

All results from laboratory investigations (haematology, serum chemistry profile and urinalysis) performed the day before to the bone marrow collection were found to be within the reference ranges established by the research institution where the oral toxicity studies were performed.

Bone Marrow Evaluation

The combined bone marrow data are summarised in Table 1. In general, bone marrow smears obtained by the collection procedure previously described showed good quality of smear preparation and adequate cellularity with numerous bone marrow particles (Fig. 1). Mature and immature haematopoietic elements as well as non-haematopoietic elements were identified.

The haematopoietic cell series were classified according to the veterinary literature (Lewis and Rebar 1979; Harvey 1984, Jain 1986, 1993).

Megakaryocytic Series (Figs 1–3)

Megakaryocytic (megakaryocytes and megakaryoblasts) elements were not included in the differential count but evaluated semiquantitatively with regards to cell number, maturation sequence and morphology. They were found to be adequate in number, maturation and morphology with more mature forms than immature forms (Figs 1–3).

Granulocytic Series (Figs 4–6)

The granulocytic series represented 47.28% of the total nucleated cell population (TNC) in females, and 47.60% in males. Granulocytic series elements comprised myeloblasts (0.29%), promyelocytes (0.67%), myelocytes (5.37%), metamyelocytes (10.51%), band granu-

Table 1. Combined bone marrow data from male and female cynomolgus monkeys

	Females Mean (%)	Males Mean(%)	M+F	
			Mean (%)	Range(%)
Granulocytic series				
Myeloblasts	0.29	0.29	0.29	0.12–0.47
Promyelocytes	0.75	0.58	0.67	0.27–1.07
Neutrophilic myelocytes	5.14	5.59	5.37	4.17–6.52
Eosinophilic myelocytes	0.02	0.02	0.02	0–0.08
Basophilic myelocytes	0	0.01	0	0–0.03
Neutrophilic metamyelocytes	9.76	10.23	10	7.27–12.68
Eosinophilic metamyelocytes	0.68	0.34	0.51	0.05–0.99
Basophilic metamyelocytes	0.08	0.06	0.07	0–0.20
Neutrophilic bands	4.30	5.15	4.73	2.51–6.88
Eosinophilic bands	0.02	0.01	0.02	0–0.08
Basophilic bands	0.01	0	0	0–0.06
Segmented neutrophils	23.79	24.31	24.05	17.10–30.56
Segmented eosinophils	1.99	1.59	1.79	1.02–2.60
Segmented basophils	0.45	0.23	0.34	0.07–0.62
Total granulocytic cells	47.28	47.60	47.44	38.11–56.37
Erythroid series				
Rubriblasts	0.34	0.33	0.33	0.06–0.61
Prorubricytes	3.38	3.28	3.34	1.60–5.07
Rubricytes	12.24	12.22	12.23	8.82–15.64
Metarubricytes	30.32	30.22	30.27	26.46–36.39
Total erythroid cells	46.28	46.05	46.17	39.55–55.11
Lymphocytic series				
Lymphoblasts	0.02	0.02	0.02	0–0.08
Lymphocytes	5.10	5.42	5.25	2.55–7.94
Total Lymphoid Cells	5.12	5.44	5.27	2.56–7.97
Plasma cells	1.49	1.45	1.47	0.67–2.26
M:E Ratio	1.02:1.00	1.03:1.00	1.02:1.00	0.75–1.29

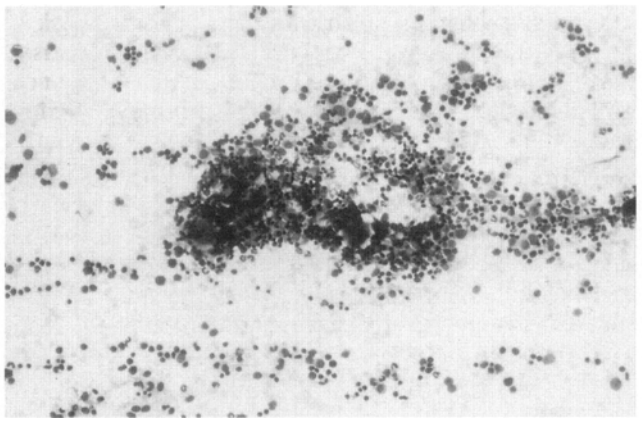

Fig. 1. Photomicrograph of a bone marrow particle from a cynomolgus monkey. May–Grünwald–Giemsa stain, 4 × objective.

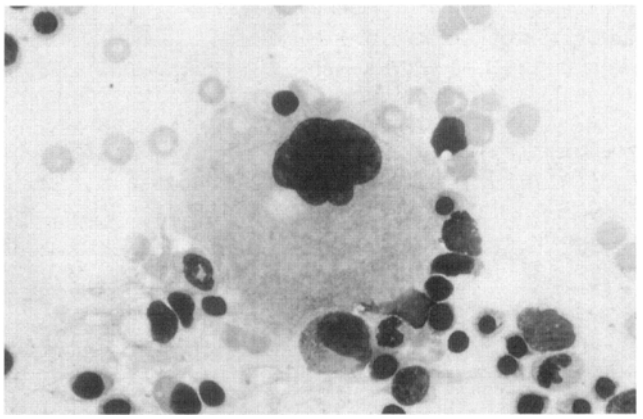

Fig. 2. Photomicrograph of a mature megakaryocyte from bone marrow of a cynomolgus monkey. May–Grünwald–Giemsa stain, 10 × objective.

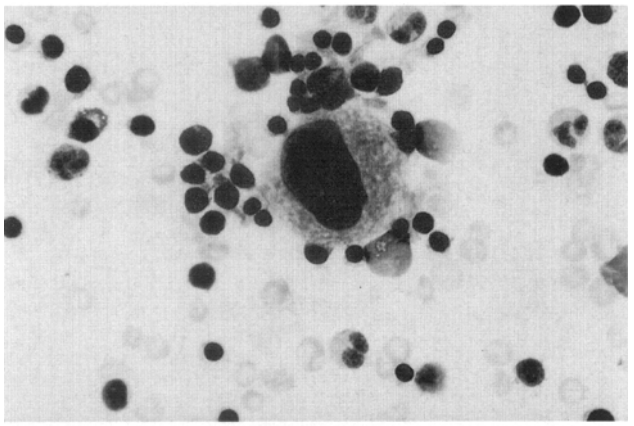

Fig. 3. Photomicrograph of an immature megakaryocyte from bone marrow of a cynomolgus monkey. May–Grünwald–Giemsa stain, 10 × objective.

locytes (4.75%) and segmented or mature granulocytes (26.18%). Myelocytes, metamyelocytes, band and mature granulocytes were further characterised as neutrophilic, eosinophilic or basophilic. The majority of the granulocytes were neutrophilic granulocytes (94%

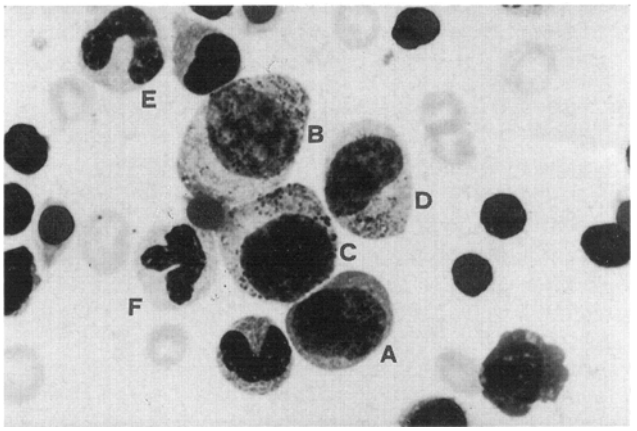

Fig. 4. Photomicrograph of a cluster of early and late stage neutrophilic granulocytes with a few metarubricytes from bone marrow from a cynomolgus monkey. A, late myeloblast/promyelocyte; B, C, myelocytes with secondary granules; D late myelocyte/early metamyelocyte; E, late metamyelocyte/early band; F, segmented neutrophil. May–Grünwald–Giemsa stain, 100 × oil objective.

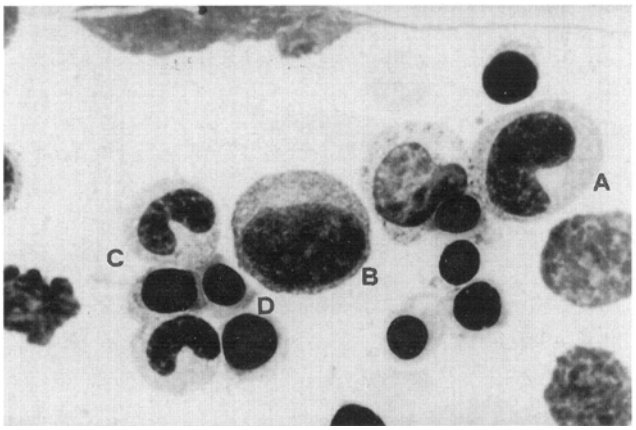

Fig. 5. Photomicrograph of early stage neutrophilic granulocytes including: A, 'Giant' metamyelocyte; B promyelocyte; C, D metamyelocytes and some metarubricytes from bone marrow from a cynomolgus monkey. May–Grünwald–Giemsa stain, 100 × oil objective.

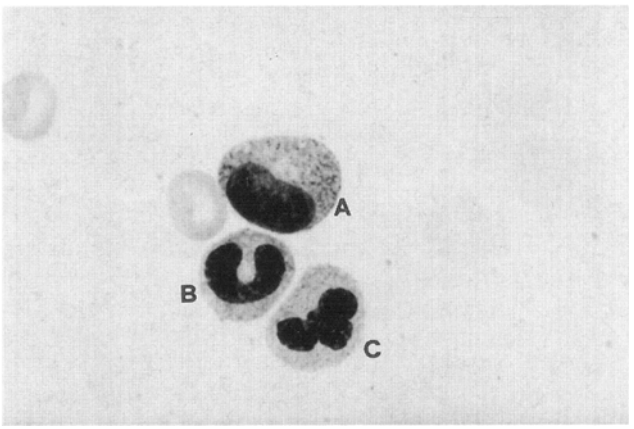

Fig. 6. Photomicrograph of later stage neutrophilic granulocytes from the bone marrow of a cynomolgus monkey. A, myelocyte; B, late metamyelocyte/early band; C, mature segmented neutrophil. May–Grünwald–Giemsa stain, 100 × oil objective.

of all granulocytes) with a lesser number of eosinophilic (5%) and only rare basophilic forms (1%).

Myeloblast (Fig. 3). The term myeloblast was applied to the most immature cell of the series. It appeared as a round cell, slightly larger than the mature granulocyte with a strongly basophilic agranular cytoplasm. The round nucleus showed an immature chromatin pattern with several nucleoli.

Promyelocyte (Figs 4 and 5). The promyelocyte measured approximately 1–1.5 times the size of the mature granulocyte. The more abundant cytoplasm was basophilic and characterised by the presence of small, reddish granules, called non-specific or primary granules. The round to oval nucleus had an open chromatin pattern and some nucleoli could be observed in the more immature forms.

Myelocyte (Figs 4–6). The myelocyte appeared smaller than the two previous forms with a lower nucleus to cytoplasmic ratio (N/C) and a lighter basophilic cytoplasm. This cell was characterised by the presence of specific or secondary granules (neutrophilic, eosinophilic or basophilic). As the myelocyte matured, the nucleus elongated and eventually, slightly edentated towards the metamyelocyte form. The chromatin pattern became coarser and the nucleoli were no longer visible.

Metamyelocyte (Figs 4–6). The metamyelocyte was the same size or smaller than the myelocyte. The cytoplasm was less basophilic and contained more of the secondary granules. The nucleus was typically kidney-shaped or indented. The chromatin pattern was coarse and more densely packed. One peculiarity noted in the metamyelocytes was the presence of some 'giant' or unusually larger forms (Fig. 5). These metamyelocytes were characterised by the same morphologic criteria but with a larger overall cell and nuclear size. This increase in the cell size could reach almost twice the size of a 'non-giant' form. Rare 'giant' forms were also oberved in the myelocyte stages. The number of cells of that particular morphology was not consistent and varied between animals. They represented a small percentage of the total metamyelocyte population and for that reason were not separated in the differential count.

Non-segmented Granulocyte (Figs 4–6). This stage was about the same size as the mature granulocyte and characterised by a nearly colorless cytoplasm containing variable numbers of secondary granules. The nucleus showed a characteristic 'U', 'C' or 'S' shape, with more mature chromatin than observed in the metamyelocyte. A slight lobulation could occasionally be observed but the lobules tended to be connected by a thick band of chromatin rather than a thin thread as in the mature form. 'Giant' forms also existed in this stage but in a lesser proportion than in the metamyelocytes.

Segmented Granulocyte (Figs 4 and 6). The mature form of the granulocytic series was characterised by a multilobulated nucleus showing a densely packed chromatin with some clumping. The clear cytoplasm contained secondary granules. Neutrophils contained reddish granules, which were very small, round, slightly eosinophilic and numerous. Eosinophils contained eosinophilic granules that appeared larger than the neutrophilic ones and round to rod-shaped and stained intensively orange–red, sometimes covering the nucleus. Basophils were rare and contained basophilic granules. These round granules stained dark purple but some were degranulated and showed clear vacuoles in a light-blue cytoplasm.

Erythroid Series (Figs 7 and 8)

The erythroid series represented 46.05% of TNC in males and 46.28% in females. Erythroid series elements comprised rubriblasts (0.29%), prorubricytes (3.34%), rubricytes (12.23%) and metarubricytes (30.27%).

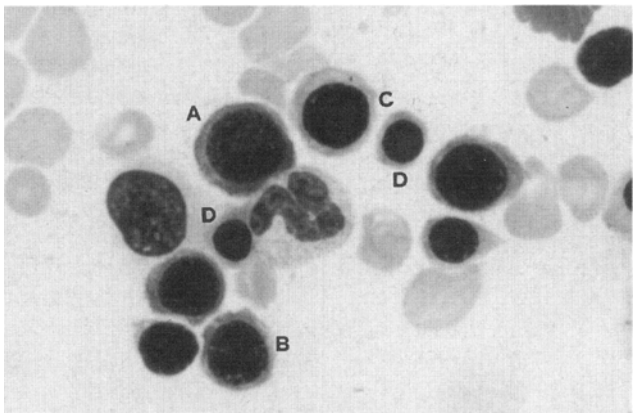

Fig. 7. Photomicrograph of early stage erythroid elements including: A, rubriblas; B, prorubricyte; C, rubricytes; D, metarubricytes, from the bone marrow from a cynomolgus monkey. May–Grünwald–Giemsa stain, 100 × oil objective.

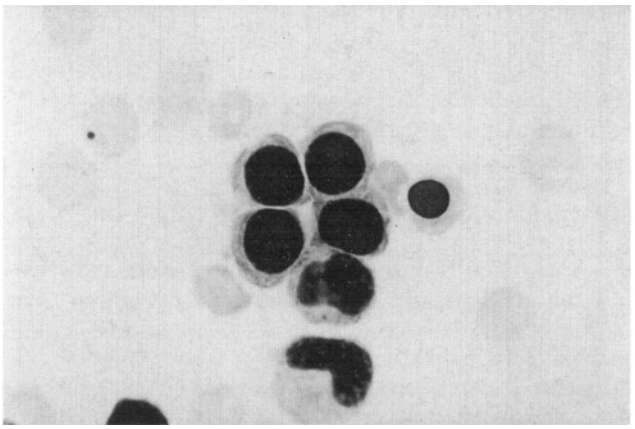

Fig. 8. Photomicrograph of a cluster of erythroid elements : four prorubricytes and one metarubricyte with two neutrophilic metamyelocytes from the bone marrow from a cynomolgus monkey. May–Grünwald–Giemsa stain, 100 × oil objective.

Rubriblast (Fig. 7). The rubriblast was the most basophilic blast of the marrow, with a high N/C ratio and a deeply basophilic cytoplasm. The nucleus was round with a reticular chromatin and contained multiple nucleoli. The nucleoli were often difficult to clearly identify due to the intense basophilic staining.

Prorubricyte (Fig. 8). The prorubricyte was smaller than the rubriblast with more of a deeply basophilic cytoplasm. The nucleus was round with more densely packed chromatin that had begun to arrange itself in a coarse-trabecular pattern. No nucleoli were visible in that stage.

Rubricyte (Figs 7 and 8). This stage was smaller than the prorubricyte and the cytoplasm had a reddish-blue coloration, due to the presence of haemoglobin. The cytoplasm was also more abundant and the nucleus had a clumped chromatin arranged in a radial structure.

Metarubricyte (Figs 7 and 8). This was the smallest cell of the erythroid series. It was slightly larger than a mature erythrocyte with an abundant, polychromatophilic to reddish cytoplasm, as the cells matured. The nucleus was small and homogenously darkly stained to pycnotic.

Polychromatophilic Erythrocytes (Reticulocytes)

Once the nucleus is extruded, the anucleated erythrocyte still retains some nucleic acid and stains more basophilic than a regular RBC and is called a polychromatophil (Harvey 1984). Polychromatophils were not counted in the differential cell count but observed in each bone marrow smear.

Lymphocytic Series

Lymphocytic cells represented 5.44% of male and 5.12% of female TNC. The series comprised mostly small lymphocytes with only rare immature lymphoid cells. Small lymphocytes were characterised by a high N/C ratio with a round to sometimes indented to 'flower-shaped' nucleus with a coarse chromatin pattern and a small amount of basophilic cytoplasm.

Plasmacytic Series (Fig. 9)

Plasmacytic cells represented 1.45% of male and 1.49% of female TNC, and were represented by mature plasma cells. Plasma cells had a darkly stained, round, eccentrically located nucleus and a moderate amount of deeply basophilic cytoplasm, with a clear perinuclear zone. Plasma cells were often unevenly distributed in clusters throughout the bone marrow smears. Mott's cells, characterised by the presence of numerous round, clear intracytoplasmic structures, were regularly observed.

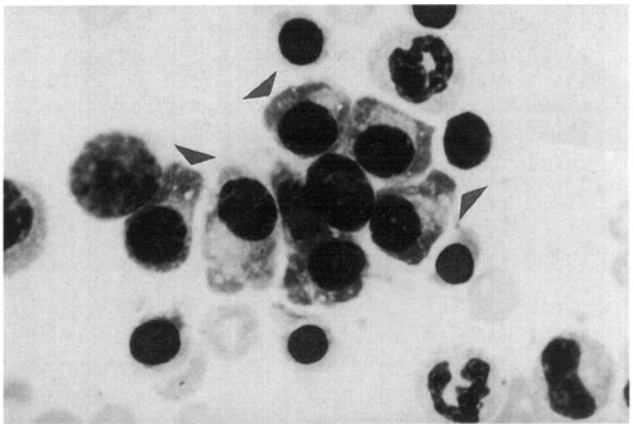

Fig. 9. Photomicrograph of a cluster of plasma cells (*arrow heads*) (admixed with segmented neutrophils and metarubricytes) from the bone marrow of a cynomolgus monkey. May–Grünwald–Giemsa stain, 100 × oil objective.

Monocytic Series

Monocytes and their precursors were quite rare and accounted for a minimal percentage of the TNC. Cells from the monocytic series were not counted separately and were included in the granulocytic series. Monoblasts were similar morphologically to myeloblasts, as were promonocytes to myelocytes.

Non-haematopoietic Elements

Other cellular elements were observed, but were not counted, and represented a very small percentage (<1%) of the TNC. Non-haematopietic elements included: connective tissue cells such as osteoblasts and osteoclasts, fibroblasts, mast cells, adipocytes, macrophages, vascular endothelial cells, etc. A few mitotic figures were also observed in most animals.

M:E Ratio

The mean M:E ratio was 1.03:1.00 for males, 1.02:1.00 for females. No statistical difference was observed between sexes ($p > 0.05$).

Discussion

The cytological composition of bone marrow in healthy, sexually mature, adult cynomolgus monkeys was studied. No significant difference was noted in the mean cell percentages nor in the M:E ratio between males and females ($p > 0.05$). The mean percentages of granulocytic and erythroid series were 47.44% and 46.17%, respectively, of the total nucleated cells, with 5.27% lymphocytes and 1.47% plasma cells. The mean M:E ratio was 1.02:1.00 with a standard deviation of 0.28. Although haematology of the macaque monkey has

Table 2. Summary of reported bone marrow data in healthy rhesus monkeys

	Porter et al. (1962)	Stasney and Higgins (1936)	Suarez et al. (1942)	Sundberg et al. (1952)	Switzer (1967)	Usacheva and Raeva (1963)	Robison and Myers (1993)
Granulocytic series (%)	48.90	52.58	61.89	51.48	53.04	58.90	43.35
Erythroid series (%)	24.60	43.74	15.80	26.10	39.12	31.01	49.05
Lymphocytic series (%)	24.60	2.53	21.57	22.42	4.49	8.60	6.00
Plasmacytic series (%)	–	–	0.62	–	2.71	1.58	1.40
M:E ratio	1.97:1.00	1.21:1.00	3.91:1.00	1.81:1.00	1.36:1.00	1.90:1.00	0.90:1.00

received a lot of attention, the literature available on the cynomolgus monkey is limited, with only rare references to the bone marrow (Hudson et al. 1976; Reiner et al 1993, Robison and Myers 1993). However, these authors did not report any normal bone marrow composition (myelogram) for this particular species, making direct comparison impossible.

Most of the work on the cellular composition of bone marrow in macaques has been performed on the rhesus monkey (*M. mulatta*) (Stasney and Higgins 1936; Suarez et al. 1942; Sundberg et al. 1952; Porter et al. 1962; Usacheva and Raeva 1963; Switzer 1967; Santiyanont et al. 1977). A summary of values obtained in this species by different authors is presented in Table 2, which shows a wide variation between authors. The mean M:E ratio varies between 1.21:1.00 (Stasney and Higgins 1936) and 3.91:1.00 (Suarez et al.1942) (approximately 70% variation), mostly secondary to the variation between percentages of erythroid cell series. Wide variations are also observed in the lymphocytic (2.53% (Stasney and Higgins 1936) to 24.60% (Porter et al. 1962) of TNC) and plasmacytic series (between 0.62% (Suarez et al. 1942) and 2.71% (Switzer 1967) of TNC).

There may be several reasons for these differences. Diet, stress, environmental conditions and general animal care (e.g., nutrition, housing) and subclinical conditions are known to influence the red blood cell picture (Jain 1986) but in most of the studies listed, there was minimal to no information on general care, environmental conditions or diet. Factors such as age and sex may be of influence (Switzer 1967), but many authors did not separate the data according to age or sex, making it impossible to determine if the variations could be due to these factors. Another important cause of variation is the bone marrow collection technique. In the rhesus monkey studies mentioned, bone marrow was collected on live, anaesthetised animals, creating more haemodilution than in a necropsy collection. Peripheral blood contamination can lead to an increased number of mature granulocytes and lymphocytes, affecting both individual counts and M:E ratios. The morphological classification of cells is also an important factor and has been shown to vary with time and author (Williams et al. 1990). Only one author (Switzer 1967) described cell morphology, but again it was not possible to be really sure that what was counted as one particular cell type was the same for each author. It could be speculated that in the study by Suarez et al. (1942) the increased number of lymphocytes could be due to confusion between metarubricytes and small mature lymphocytes, as commonly described (Jain 1986).

We decided to compare our data more closely with the study by Switzer (1967) as it represented the most complete report available in this species. A slight difference was noted in the mean M:E ratio in the study reported by Switzer when compared to the present study (1.36:1.00 vs 1.02:1.00), due to a greater percentage of the granulocytic series (53.04% vs 47.24%) and a concomitant smaller number of erythroid cells (39.12% vs 47.33%). Switzer also reported fewer lymphocytes, but plasma cells showed a slightly higher percentage (2.72% vs 1.47%). Differences between the studies were minimal and quantitatively all below 25%. Considering that the bone marrow differential count is a manual procedure, very much dependent on an individual's interpretation, and therefore liable to greater variation than other techniques, the overall impression was that there was an acceptable correlation between the bone marrow composition of the rhesus and the cynomolgus monkey.

Non-haematopoietic elements observed in cynomolgus monkeys are also observed in other animal species (Lewis and Rebar 1979; Harvey 1984; Tyler and Cowell 1989). It is important to know that they exist in small percentages as these elements can be the first and/or only elements to increase in number or to show morphological changes following a haematotoxic effect (Rebar 1993). For example, macrophages and their phagocytic activity can be increased in bone marrow necrosis (Irons 1991).

Conclusion

In conclusion, bone marrow collection at necropsy was found to be an acceptable method, yielding good quality and good cellularity of specimen. May–Grünwald–Giemsa staining gave good cellular detail on both haematopoietic and non-haematopoietic elements. Cytological examination of the bone marrow appeared to be a rapid and useful method that provided good detail on the morphology and the maturation of haematopoietic cell

lines. Data obtained from this study showed that the bone marrow composition of the cynomolgus monkey was similar to that of the rhesus monkey. The rapid development of haematopietic growth factors and cytokines as potential therapeutic agents may lead to a greater need for complete examination of bone marrow. Cytological examination should be added to clinical haematology, histopathology, flow cytometry, ferrokinetics, bone marrow culture and analysis of stem cell populations to better determine the mechanisms of compound toxicity and to increase the knowledge in the field of haematotoxicity of the cynomolgus monkey.

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