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# The Megakaryocyte DNA Content and Platelet Formation After the Sublethal Whole Body Irradiation of Rats

By Gunnar Tanum

**The DNA content of rat bone marrow megakaryocytes (MK) was studied by Feulgen photometry, following whole body irradiation with 2 Gy. The DNA measurements were preceded by acetylcholinesterase staining to avoid missing the smaller 2N–8N MK. The number of 2N–8N MK declined immediately following irradiation, whereas the number of 16N–64N MK remained normal for 4 days before decreasing. The number of 2N–8N and 16N–64N MK reached minimum around days 7 and 10, respectively, and thereafter increased to supranormal values at days 14 and 20, respectively. Platelet production, measured by $^{35}$S incorporation into platelets, increased during the first 4 days, then decreased to minimum about day 10. A rise to supranormal values was present at day 20. All values were about normal 30 days after exposure. The observed pattern may be explained as follows: Most of the 16N–64N MK survive the applied dose and maintain their ability to produce platelets. Some of the 2N–4N and 8N MK survive irradiation and transform into platelet-producing MK. No influx of cells from the MK stem cell compartment into the MK compartment can be observed before day 7 after irradiation. One explanation for this time lag may be that thrombocytopenia, which does not occur before then, is an essential stimulus for MK stem cell activation.**

SEVERAL STUDIES have shown that a single dose of whole body irradiation reduces the number of megakaryocytes (MK) and causes thrombocytopenia, with nadir around day 10 and recovery 2–3 wk following exposure.[1–9] Mature MK are relatively radioresistant and immature MK radiosensitive.[7] There are few, if any, reports on changes in DNA content of MK after irradiation. This has been studied in the present report, together with the MK number, platelet production, and platelet counts.

## MATERIALS AND METHODS

Twenty-six male inbred Lewis rats, (supplied by Møllegaard Hansen Avlslab A/S, Ejby, Denmark), 2–2.5 mo old, were used for the study. The animals were kept under controlled environmental conditions. Irradiation was performed by a Stabilipan (Siemens A.G., Erlangen, W. Germany) roentgen unit at 220 kV and 20 mA with filter 1 Cu. The animals were given a single dose of 2 Gy (200 rad) whole body irradiation. Groups of 3 animals were sacrificed and examined for their blood platelet number, platelet production, and DNA content of bone marrow MK at days 2, 4, 7, 10, 14, 20, and 30 following irradiation. Five unradiated animals were used as normal controls.

### *Platelet Counts*

Under ether anesthesia, 0.1 ml blood was collected from a femoral vein and mixed with 1 ml Isoton II (Coulter Electronics Ltd., Luton, England). The platelets were counted by a Coulter Counter (Coulter Counter S+, Coulter Electronic Inc., Hialeah, FL). The blood platelet counts performed by the Coulter Counter were compared to the counts performed by phase-contrast microscopy and Ultra-flo 100 (Clay Adams Lab. Instr., Parsippany, NJ), the latter machine being adapted to measurements of small platelets typical for rats. All three methods gave counts within the same range. The Coulter Counter generated complete size distribution curves, indicating that the machine did count the small blood platelets and, on the other hand, did not count small erythrocytes as platelets. The blood platelet counts performed by the Coulter Counter thus seem to be reliable for the strain of rats used for the present study.

### *Platelet Production*

The platelet production was determined by measuring the incorporation of $^{35}$S-sodium sulfate into platelets, as described by Dziewatkowsky[10] and Odell et al.[11] The platelets were labeled in vivo by an intraperitoneal injection of 8.3 megabequerel $^{35}$S-sodium sulfate dissolved in sterile 0.9% NaCl, given 42 hr before the animals were sacrificed. Blood was collected from the aorta after laparotomy. Two milliliters platelet-rich plasma was washed in 1% ammonium oxalate and 0.9% NaCl, resuspended in Lumagel (Lumac System AG, Basel, Switzerland), and the $^{35}$S activity was counted in a liquid scintillation system (Mark III, Searle Analytic Inc., Des Plaines, IL). The $^{35}$S incorporation into platelets was calculated as a percentage of the total injected $^{35}$S dose:

$$\frac{\text{Radioactivity/platelet} \times \text{Total platelet number}}{\text{Radioactivity of injected dose}} \times 100.$$

In pilot studies, plasma radioactivity was measured at various intervals following $^{35}$S injection in 3 irradiated and 3 control animals to monitor for the possibility of an abnormality in sulfate metabolism in the irradiated animals compared to the controls. The curves for plasma radioactivity were about parallel, indicating that sulfate metabolism was similar for both groups.

### *MK Number and DNA Content*

The method for measuring DNA of rat bone marrow MK has been thoroughly discussed previously,[12] especially as to the reliability of the method and its implications compared to previous MK detection methods. Briefly, the bone marrow was completely removed from both femur diaphyses. All MK on one smear made from 1.2 μl bone marrow from each animal was identified by *S*-acetylcholiniodid staining. In rodents, this stain binds to acetylcholinesterase and colors the platelets and MK brown, including the low ploidy MK, which are otherwise difficult to identify. Other bone marrow cells remain unstained. All MK on the smear were identified and numbered, and the MK number per microliter bone marrow could thus be calculated. The position of the MK was recorded by a

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grade system. For photometry, the smear was decolorized, as described by Jackson,[13] and restained with Feulgen stain.[14] In each smear, a representative sample of 70 MK was examined for its DNA content. This sample was randomly selected by drawing numbers allotted to all MK in the smear. Photometry was performed by a scanning microspectrophotometer (SMPO Zeiss, Oberkochen, W. Germany). A wavelength of 570 nm was used, corresponding to the stained components absorption maximum, with objective ×100 immersed in glycerol. For 2N reference value, 25 segmented granulocytes from each smear were measured. The DNA values of the various ploidy classes were calculated on the basis of the observed granulocyte diploid value. Each ploidy class was defined according to an internationally recognized convention,[15] where all MK are placed into ploidy classes without any interploidy compartments.

## RESULTS

In the controls, the number of blood platelets was $0.859 \pm 0.055 \times 10^6/\mu l$ and the $^{35}S$ incorporation into platelets was $5.0 \pm 0.3 \times 10^{-3}\%$ of the injected dose. In the irradiated animals, the blood platelet counts remained normal for 4 days, before declining to a minimum of about 30% of normal 10 days following exposure (Fig. 1A). The platelet counts rose to supranormal levels (about 130% of normal) at day 20. The $^{35}S$ incorporation into platelets increased to about 170% of normal after 2 days, then decreased to about 10% of normal at day 10, and increased again (about 270% of normal) at day 20, before normalizing (Fig. 1B).

The total number of MK remained normal for 2 days, then declined to minimum (about 30% of normal) after 7 days, and rose to supranormal level (about 190% of normal) at day 20 (Fig. 1C). In Fig. 2, the MK have been placed into 3 main ploidy compartments: 2N + 4N, 8N, and 16N + 32N + 64N. All together, 1,824 MK were recorded. The number of MK within the 2N–4N compartment declined to nearly zero during the first 4 days following exposure. Between days 7 and 10, the number of 2N–4N MK started to increase, reached maximum (about 470% of normal) at day 14, and stayed high for several days before normalizing. The number of 8N MK followed the same pattern, decreasing to nearly 0 after 7 days, then increasing to maximum (about 135% of normal) 14 days after irradiation. The number of MK within the 16N–64N compartment slightly increased the first 2 days, then declined to minimum (about 10% of normal) after 10 days, before increasing to maximum (about 230% of normal) at day 20. All values were about normal 30 days following irradiation exposure (Figs. 1 and 2).

## DISCUSSION

In accordance with previous studies[2,5] the blood platelet counts remained normal for 4 days, then declined to minimum about day 10, before reaching a

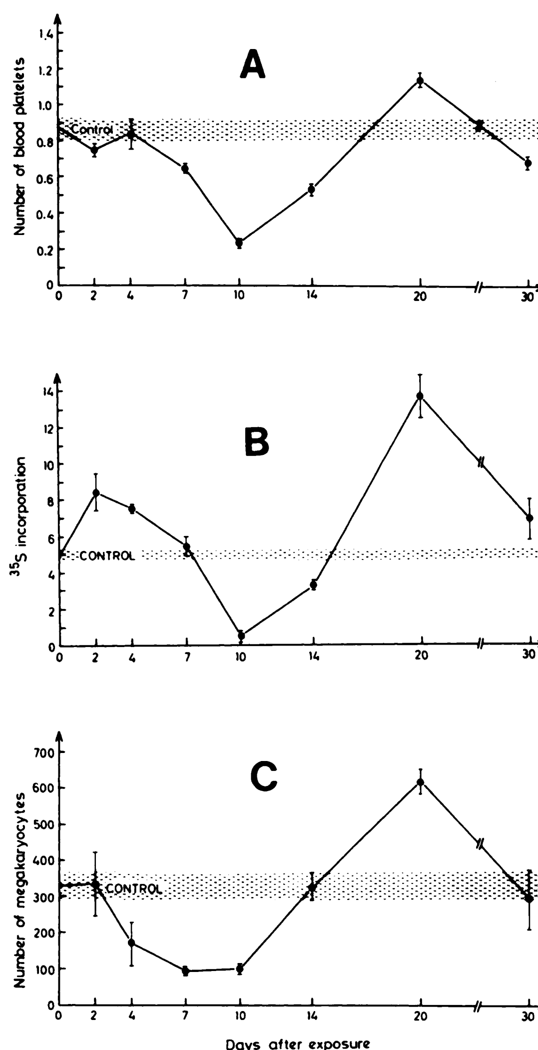


**Fig. 1. Blood platelet counts × $10^6/\mu l$ (A), $^{35}S$ incorporation into platelets expressed as percent of total injected dose × $10^{-3}$ (B), and total number of megakaryocytes/μl femoral bone marrow (C) from rats at various intervals after whole body irradiation with 2 Gy. The days shown on the abcissa represent the time at which the rats were sacrificed. Each point represents the mean of 3 animals ± 1 SE, except for the controls, which include 5 animals.**

rebound thrombocytosis about 20 days after a sublethal exposure of irradiation (Fig. 1A). The platelet production varied proportionally to the platelet counts, except that the platelet production was stimulated to about 160% of normal during the first 4 days after irradiation (Fig. 1B). Finding normal platelet counts at the same time as platelet production is increased indicates increased removal of platelets from circulation during this period. This is probably not caused by a direct effect of irradiation on the platelets, because they have been found to be highly radioresistant.[16]

The main findings in the present study are the variations observed in MK number within the different ploidy classes following irradiation. In contrast to some

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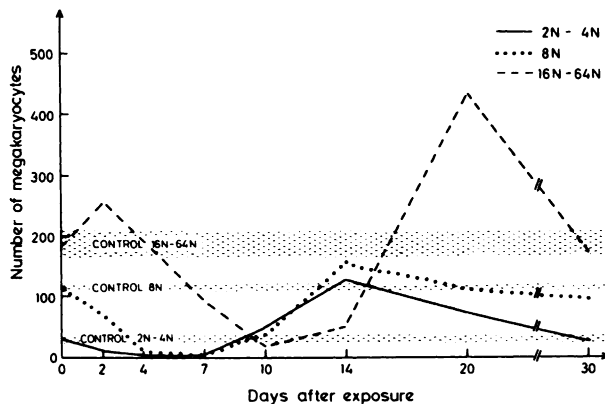


**Fig. 2. The number of megakaryocytes within the 2N–4N, 8N, and 16N–64N ploidy classes per microliter bone marrow from femur of rats at various intervals after whole body irradiation with 2 Gy. The days shown on the abcissa represent the time at which the rats were sacrificed. Each point represents the mean of 3 rats ± 1 SE, except for the controls, which include 5 rats.**

investigators,[6,8] but in accordance with others,[2,3,9] we did not find any significant initial rise in total MK number (Fig. 1C). One reason why an initial increase in total MK number has been observed by others[6,8] may be that the number of 16N–64N MK increased, as found in this study. However, the total MK number does not change significantly, because the number of 2N–8N MK, which probably have not been distinguished in previous studies, decreases correspondingly (Fig. 2).

The slight increase of the number of 16N–64N MK at day 2 corresponds with the increased platelet production at the same time (Figs. 1B and 2). This indicates that 16N–64N irradiated MK are able not only to produce platelets, but also to increase their platelet production. The number of 16N–64N MK dropped after day 2, with nadir at day 10. As the lifespan of rat MK is about 3 days,[17,18] the low gradient slope of the 16N–64N MK curve (Fig. 2) indicates that either the surviving MK with lower DNA content are transformed into higher ploidy classes or there exists a pool of radioresistant, resting, mature MK that is slowly activated to produce platelets, or both. This means that DNA replication and/or cytoplasmic maturation can occur shortly after irradiation.

In contrast to the 16N–64N MK, the 2N–4N and 8N MK nearly disappeared shortly after exposure and stayed low until day 7 (Fig. 2). From this study, one cannot decide how many of the 2N–4N and 8N MK are killed by irradiation and how many develop into higher ploidy classes during the first days after exposure.

The regeneration of the MK population started after day 7 by proliferation of the 2N–4N MK (Fig. 2). This regeneration has to be caused by influx of cells from the MK stem cell population, because the MK themselves are not capable of mitosis. However, the issue of whether or not 2N MK that have acquired the ACHE marker are capable of mitosis has not been finally resolved. At day 14, the number of 2N–4N and 8N MK reached the peak level, whereas the number of 16N–64N MK still remained low (Fig. 2).

At this time, the platelet production was about 70% of normal (Fig. 1B), which indicates that some of the MK with a DNA content less than 16N (probably the 8N) participate in platelet production during the recovery phase. The number of 16N–64N MK reached peak value at day 20 (Fig. 2), when the platelet production was also increased (Fig. 1B). This is reasonable, as increasing the mean MK DNA content is one way the MK population increase their platelet production.[17,18]

The observed findings may be explained as follows: Most of the 16N–64N MK survive the irradiation dose applied and maintain their ability to produce platelets. Some of the 2N–4N and 8N MK survive irradiation and later transform into platelet-producing MK. No influx of cells from the MK stem cell compartment into the MK compartment can be observed until days 7–10 after irradiation (Fig. 2). One explanation for this time lag might be that the number of dividing committed stem cells is much reduced by irradiation and that thrombocytopenia, which does not occur before day 7, is an essential stimulus to activate the uncommitted stem cells to proliferate.

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