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Short Note

Repopulation of Irradiated Mice with Medicinally Stimulating Cells and Infection with Moloney Sarcoma Virus Have a Profound Effect on Regeneration of Splenic Megakaryocytes

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The megakaryocyte (Mg) numbers per cross-sectioned spleen slides were arbitrarily evaluated in untreated Balb/cann and C57Bl inbred mouse strains, in sublethally (600 R) and lethally (850 R) irradiated mice and in mice repopulated with syngeneic bone marrow or marrow stromal cell cultures at various time intervals. The impact of Mu-MSV infection or of Mu-MSV induced sarcoma on spleen Mg was also evaluated. Spleens of control mice of both strains harbored numerous Mg. Following sublethal irradiation, severe atrophy of the spleen resulted in the disappearance of Mg from day 6 lasting to day 16. Repopulation with marrow cells lead to the reappearance of Mg by day 8, their proliferation as evidenced by mitosis and full recovery of the population on day 24. Bone marrow stromal cell cultures failed to repopulate irradiated spleens with Mg. C57Bl mice irradiated with 850 R and repopulated with syngeneic bone marrow also recovered their Mg population, although a full recovery required a longer time. Mg in the spleen of Moloney sarcoma virus infected mice or in spleen of mice which developed tumours following local administration of Mu-MSV greatly increased in number by day 18 post infection, and their numbers exceeded those of normal animals, thus the Mu-MSV can be considered as having a positive effect on spleen Mg in rodents.

Key words: Megakaryocytes, mice, Moloney sarcoma virus, spleen.

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Megakaryocytes, the platelet-generating cells, are a component of hematopoietic bone marrow. Mice and rats are species which also harbor these cells in the spleen (DA SILVA *et al*. 2012). Several publications have shown the presence of splenic megakaryocytes in mice and changes in numbers due to infection or tumors (CAJUEIRO *et al*. 2017; RAY 2000). However, numerous histological textbooks do not record this fact. Recently edited textbooks of histology do not mention megakaryocytes as a cellular component of the spleen (JUNQUEIRA & CARNEIRO 2003; GARTNER & HIATT 2007; SAWICKI & MALEJCZYK 2012). ROSS and ROMRELL (1989) noted that megakaryocytes occur in the spleen of certain species, such as rodents and cats, but not in humans. JAKÓBISIAK (1993) stated that hematopoiesis, including platelet formation, occurs in fetal human spleen but only in some pathological conditions, such as anemia, sever infections and lymphomas in adult humans there called splenic bone-marrow metaplasia. In the adult murine spleen hematopoietic and platelet production can be triggered following intravenous administration of hematopoietic stem cells or by

lymphocyte mitogens, as evidenced by "spleen nodule" formation (TILL & MCCULLOCH 1961; CURRY & TRENTIN 1967; JENKINS *et al.* 1969; WŁODARSKI *et al*. 1974).

This report describes the presence of megakaryocytes in the mouse spleen following a range of treatments. The objective of the present study was to evaluate semi-quantitatively the impact of irradiation and repopulation with fresh bone marrow cells on megakaryocytes in the spleen at various stages of post irradiation recovery and the effect of Moloney sarcoma virus (MSV) infection on the megakaryocyte population in the spleen.

Materials and Methods

Animals

Inbred Balb/cann, C57Bl/6N and C57Bl/6JBg mice of both sexes, aged 11-14 weeks bred in the vivarium of the National Institute of Dental Research, NIH, Bethesda, USA were used in the experiments. At that time no written permission for using laboratory animals was required. Nevertheless animals were handled in accordance with the guidelines and protocols approved for these experiments by NIDR, Bethesda.

Irradiation: whole body X-ray irradiation at a dose of 600 R or 850 R was administered using a Philips RT therapeutic machine (200 kV, filter 0.5 mm Cu, tube 20 x 24 cm, SD 50 cm, HVL – 1.1 mm Cu).

Repopulation of irradiated animals

Three to four hours post irradiation, animals were infused via a tail vein with $1x10^6$ nucleated fresh bone marrow cells from syngeneic untreated donors. Bone marrow cells were obtained by flushing out femoral marrow cavities, and the yield was measured using a Coulter cell counter. One million nucleated cells, suspended in 0.2 ml of Parker 199 cell culture medium were injected into the tail vein. In some instances, stromal bone marrow cell cultures, grown for 14 days *in vitro*, were similarly injected at a dose of $0.5x10^6$ viable cells. This set served as an additional control of stress conditions on splenic megakaryocytes.

Balb/cann mice bearing the Mu-MSV-induced tumor

Animals were injected with MSV 129A stock solution into shank muscles and with 2.5 mg of cortisone acetate, administered subcutaneously. Within 10 days all animals developed a tumor at the site of virus administration which grew progressively with time, reaching a peak on day 22-24th. Then blood was collected, and 0.2 ml of the serum obtained was injected into a tail vein of intact mice. Their spleens were examined 18 days post serum administration.

Histological procedure

Animals were killed by cervical dislocation; their spleens were fixed in formalin and embedded in plastic; 2.0 μm sections were stained with 0.5% toluidine blue. The frequency of megakaryocytes per section was arbitrarily evaluated at 200x magnification using an Olympus CX41 microscope.

Scoring of megakaryocyte numbers in splenic sections

Megakaryocyte numbers per cross-sectioned spleen slide were arbitrarily and approximately evaluated by estimation of their presence in several microscopic fields. The score was classified as: None (absence of megakaryocytes); Single – a few megakaryocytes on the section); Few – megakaryocytes appeared scattered on the section, (approximately 1 cell per field); Moderate – megakaryocytes evenly distributed across the section, more than 1 cell per field; Numerous – a few megakaryocytes present in the majority of fields; Large quantity – densely packed megakaryocytes in numerous fields.

Results and Discussion

Megakaryocyte numbers in the spleens of two inbred strains Balb/cann and C57Bl of untreated mice were first assessed following irradiation with 600 R and 850 R and then after repopulation with normal syngeneic bone marrow cells, as well as following infection with oncogenic Mu-MSV virus. Full details of experimental conditions are listed in Table 1.

Megakaryocytes were identified according to morphological criteria: large cells with diameter 50-100 μm having a polyploid lobulated (3-7 lobes) nucleus and pale cytoplasm sometimes exhibiting a delicate meshwork. These cells were predominantly located in the red pulp, close to the spleen capsule.

Spleens of untreated mice of both strains harbored numerous megakaryocytes. Following irradiation with 600 R, severe atrophy of the spleen was observed, and from day 6 until day 16 post irradiation no megakaryocytes were found. Repopulation of such animals with $1x10^6$ nucleated healthy bone marrow cells led to the reappearance of megakaryocytes by day 8. Numerous megakar-

Table 1

Experimental conditions and megakaryocyte numbers in the spleens of two inbred strains Balb/cann and C57Bl of untreated mice, following irradiation with 600 R and 850 R and after repopulation with normal syngeneic bone marrow cells, as well as following infection with oncogenic Mu-MSV virus

Strain of mice	Experimental condition	Repopulation with syngeneic bone marrow (B.M.)	No of specimen	Duration (days)	Estimation of megakaryocyte frequency on spleen sections
Balb/c	Intact control	No	8	–	Numerous in all cases
	600 R	No	8	6-16	Absent
	600 R	$1x10^6$ B.M.	6	6	Single ghost
	600 R	$1x10^6$ B.M.	2	8	Numerous mitoses
	600 R	$1x10^6$ B.M.	2	24	Numerous
	600 R	$0.5x10^6$ stromal cells	4	6	None, single
Balb/c	MSV tumour bearers	No	2	15	Numerous
		No	6	20	Large quantity
Balb/c	MSV tumour – bearing serum i.v. infusion	No	3	18	Large quantity
C57Bl	Intact control	No	5	–	Numerous in all cases
	850 R	$1x10^6$	6	24	Few/moderate
	850 R	$0.4x10^6$	3	32	Moderate
	850 R	$1x10^6$	8	45	Moderate/Numerous, mitoses

yocytes, some being in metaphase stage, were identified and on 24 days post-repopulation (Fig. 1), they were still numerous. This indicates that the recovery of megakaryocytes is relatively rapid and requires about 8 days of development from marrow stem cells. Bone marrow stromal cells failed to repopulate irradiated spleens with megakaryocytes (Fig. 2).

C57Bl mice, irradiated with 850R and repopulated with syngeneic bone marrow also recovered their megakaryocyte population, although a full recovery required more time and they reached moderate/numerous numbers by day 45. Megakaryocytes in the spleen of Balb/c mice, bearing Mu-MSV-induced tumours were numerous, and with progression of tumor growth their numbers increased greatly. Megakaryocyte numbers in Balb/cann mice infected by intravenous administration of serum obtained from Mu-MSV-bearing mice produced a marked increase in splenic megakaryocytes by day 18 post injection (Fig. 3). This route of Mu-MSV administration does not lead to the formation of sarcoma, but such serum, if administered into shank muscles, resulted in local development of sarcoma in the same intensity as stock virus did.

Megakaryocytes comprise a consistent component of the spleens of normal Balb/c and C57Bl mice. Their numbers vary depending of pretreatment pathology. Following irradiation, splenic atrophy leads to the elimination of megakaryocytes within 6 days. Repopulation with $1x10^6$ untreated syngeneic marrow cells results in the reappearance of megakaryocytes after 8 days, and by 24 days post-repopulation their numbers are back to normal numbers. Hematopoietic stem and progenitor cells are able to produce megakaryocytes *in vitro*, in response to a specific growth factor – thrombopoietin (IVETIC *et al.* 2016). It is likely that these cells, present in bone marrow, can proliferate and differentiate when infused *in vivo* into irradiated recipients. Alternatively, according to a novel theory by CAPLAN 2017, the infused hematopoietic stem cells can trigger proliferation and differentiation of the *in situ* resident progenitor of the megakaryocyte lineage. We failed to conclude whether megakaryocytes differentiate from the stem cells within the spleen or if they are donor-derived. Repopulation with stromal bone marrow cells grown *in vitro* failed to restore the megakaryocyte population in irradiated mice. C57Bl mice irradiated with 850R and repopulated with syngeneic bone marrow also recovered their megakaryocyte population but at a slower rate, reaching a normal level on day 45 (Fig. 4).

The effect on splenic megakaryocytes and on mast cells resident in the spleens of Balb/cann mice irradiated with 800R and repopulated with bone marrow differs greatly. Post-irradiation atrophy of the spleen and lymph nodes in Balb/cann mice was not followed by depletion of resident tissue mast cells (WŁODARSKI 1987; 1988), as is the case with megakaryocytes. No increase in splenic

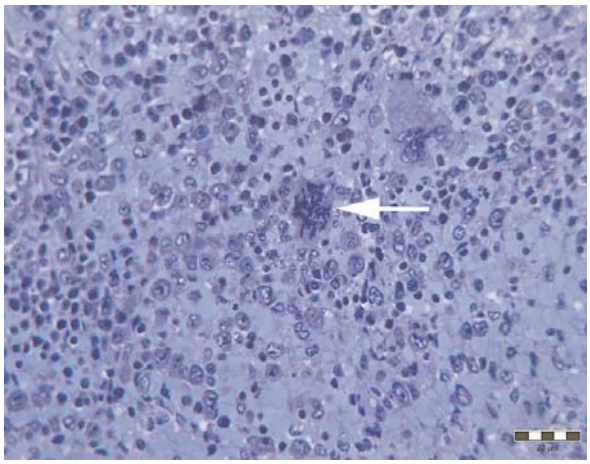

Fig. 1. Mitoses (white arrow) in megakaryocytes in mice irradiated with 600 R and repopulated with syngeneic bone marrow, 8 days post repopulation. Scale bar = 20 µm.

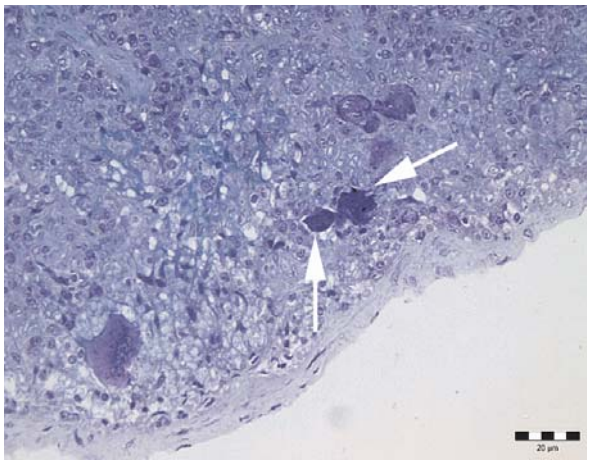

Fig. 2. Degeneration and depletion (white arrow) of megakaryocytes in mice irradiated with 600 R and repopulated with stromal marrow cells, 6 days post infusion. Scale bar = 20 µm.

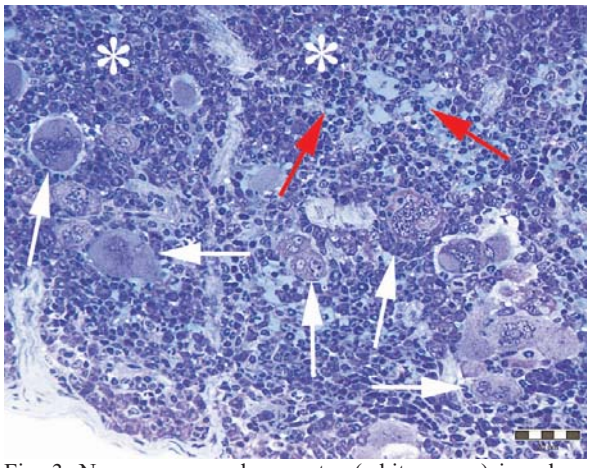

Fig. 3. Numerous megakaryocytes (white arrow) in spleen (* – white pulp; red arrow – red pulp) of MSV infected mice, 24 days post MSV infection. Scale bar = 20 µm.

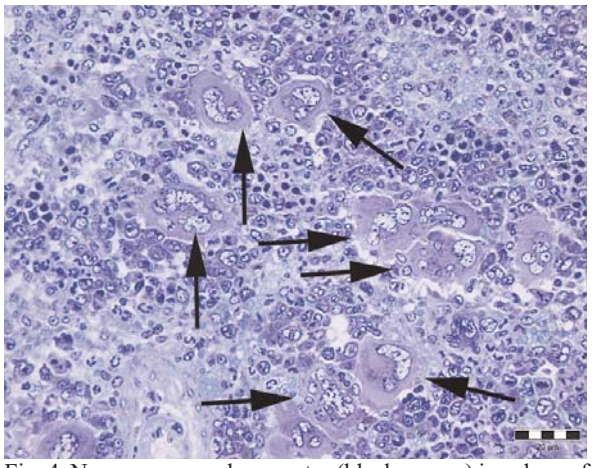

Fig. 4. Numerous megakaryocytes (black arrrow) in spleen of C57/Bl mice irradiated with 850R and repopulated with syngeneic bone marrow 45 days post repopulation. Scale bar = 20 µm.

mast cells was seen following post irradiation repopulation, therefore it can be concluded that 800 R does not destroy tissue mast cells.

The presence of Mu-MSV tumours greatly increases megakaryocyte numbers in the spleen. In both, development of the tumor induced by this virus and also intravenous administration of the virus results in a great increase in splenic megakaryocytes after 18-20 days . A similar observation was recently reported by SEONG *et al.* (2016). They found a substantial increase in the number of megakaryocytes in spleens 5-9 days post- oral infection of mice with bovine diarrhea virus, suggesting that the increased number of megakaryocytes may be a direct result of BDVD infection.

Our experiments add Mu-MSV infection to the list of agents having a positive effect on spleen megakaryocyte numbers in rodents. Whether this conclusion could be prescribed to osteoblasts, rapidly proliferating following Mu-MSV infection (WŁODARSKI *et al*. 1979; WŁODARSKI *et al*. 2007) and able to secret interleukin 9, cytokine promoting megakaryopoiesis (XIAO *et al*. 2017) or growth factors and bone matrix proteins released by sarcoma induced by this virus (WŁODARSKI *et al*. 2007), remains to be elucidated.

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