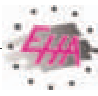

Human cell engraftment after busulfan or irradiation conditioning of NOD/SCID mice

Elodie Robert-Richard
Cécile Ged
Jacqueline Ortet
Xavier Santarelli
Isabelle Lamrissi-Garcia
Hubert de Verneuil
Frédéric Mazurier

Human hematopoietic stem cell (HSC) xenotransplantation in NOD/SCID mice requires recipient conditioning, classically achieved by sublethal irradiation. Pretreatment with immunosuppressive and alkylating agents has been reported, but has not been rigorously tested against standard irradiation protocols. Here, we report that treatment of mice with a single dose (35 mg/kg) of Busilvex®, an injectable form of busulfan, enables equivalent engraftment compared to 3.5 Gy irradiation. Mice treated with two doses of 25 mg/kg to reduce busulfan toxicity showed increased chimerism. Busulfan conditioning and irradiation resulted in comparable sensitivity of HSC detection as evaluated by limiting dilution analysis.

Key words: NOD/SCID, transplantation, SCID repopulating cell assay, busulfan, conditioning

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From the INSERM E217, Bordeaux, F-33076; Université V. Segalen Bordeaux 2, Bordeaux, F-33076, France (ER-R, CG, IL-G, HdV, FM); CHU de Bordeaux, Hôpital Saint André, Service de Radiothérapie, Bordeaux, F-33000, France (JO); ESTBB, Université V. Segalen Bordeaux 2, Bordeaux, F-33076, France (XS).

Correspondence:
Frédéric Mazurier, INSERM E217, Université V. Segalen Bordeaux 2 146 rue Léo Saignat, 33076 Bordeaux cedex, France
E-mail: mazurier@u-bordeaux2.fr

In order to gain insight into the biology of the human hematopoietic stem cell (HSC) compartment and to understand the mechanisms underlying malignant transformation, *in vivo* models for human repopulating cells are required. Available xenotransplantation models use immune-deficient mouse recipients such as NOD/SCID,[1] RAG/γc,[2] NOD/SCID/β2m,[3] NOD/SCID/γc[4,5] and NOD/RAG/Prf.[6] The NOD/SCID assay, which detects SCID repopulating cells (SRC), is a powerful tool that enables characterization of human HSC capable of sustained multilineage differentiation, and also allows evaluation of HSC homing. The residual immunity of the mice can be further depleted by additional pretreatment with antibodies directed against NK cells or macrophages.[7-9] Nevertheless, despite their profound immune-deficiency, NOD/SCID mice require a preparative regimen in order to permit engraftment of human hematopoietic cells. Standard conditioning protocols involve sublethal irradiation of the mice (up to 4 Gy), which results in depletion of murine hematopoietic cells and increased concentrations of growth factors and chemoattractants in the murine bone marrow microenvironment prior to transplantation. However, access to a source of radiation is limited for many laboratories.

Clinically since the early 1980s, total body irradiation (TBI) prior to human HSC transplantation has been progressively replaced by the use of a combination of chemotherapeutic agents such as cyclophosphamide and busulfan. Cyclophosphamide is highly immunosuppressive, whereas busulfan, a myeloablative alkylating agent, functions similarly to TBI by depleting non-cycling primitive stem cells.[10,11] In mouse studies, recipients of murine bone marrow cells exposed to busulfan showed a decrease in peripheral blood counts, marrow cellularity, stem cell content, self-renewal capacity, and long-term survival.[12-14] Some xenotransplantation studies have reported the use of busulfan to condition newborn immune-deficient mice[6,15] in order to achieve human HSC engraftment. However, these studies did not provide a systematic comparison of busulfan versus irradiation conditioning with regards to SRC detection in adult mice. Here, we report the establishment of a busulfan conditioning protocol for NOD/SCID mice and demonstrate that busulfan treatment provides equivalent SRC detection efficacy compared to the standard irradiation regimen.

Design and Methods

NOD/SCID mouse repopulation

Cord blood cells were obtained according to procedures approved by Etablissement Français du Sang-Aquitaine (France). CD34$^+$ cell enrichment (92 to 99%) was achieved by positive selection (EasySep™; Stem Cell Technologies, Vancouver, BC, Canada). Cells were transplanted into 7- to 10-week old mice using intravenous injection for primary mice and intra-osseous injection for secondary mice. Prior to transplantation, mice received preparative regimens consisting of either irradiation (3.5 Gy at 0.8 Gy/mn; 6MV Clinac 2100C, Varian, Palo Alto, CA, USA) or intraperitoneal injection of Busilvex®, an injectable form of busulfan (Pierre Fabre, Boulogne, France). All mice were also given a single intraperitoneal injection of 75 μg anti-CD122 antibody generated from the TM-β1 hybridoma cell line.[16] Bone marrow (BM) samples were aspirated from the right femur.[17] Human cell engraftment

was assessed by flow cytometry. BM (samples from femurs, tibias and pelvis) from primary mice was frozen prior to secondary transplantation.

Flow cytometry

Flow cytometric analysis was performed using a FACScalibur™ (Becton Dickinson, San Josè, CA, USA). Isotype controls were mouse immunoglobulin-G conjugated to phycoerythrin (PE), allophycocyanin (APC) (both from BD), PE-cyanin 5 (PC5), or PC7 (Beckman-Coulter, Fullerton, CA, USA). CD34 expression was assessed using anti-CD34-PC5. Human grafts in mice were assessed using anti-CD45-PC7, anti-CD19-PE, anti-CD33-APC, anti-CD36-PE and anti-glycophorin A (GPA)-APC antibodies.

Progenitor assays

Human colony-forming cells from the BM of engrafted mice were assayed as previously described[17] with minor modifications. Methylcellulose (Stemα.I, Stem alpha SA, Saint Clément les Places, France) was supplemented with 10% human plasma, interleukin-3 (25 ng/mL), granulocyte-monocyte colony-stimulating factor (GM-CSF) (25 ng/mL), stem cell factor (50 ng/mL) and erythropoietin (3 U/mL).

Statistical analysis

Data are presented as the mean ± SEM. The significance of differences between groups was determined by the Student's t-test. SRC frequencies were calculated using L-calc software (Stem Cell Technologies).

Results and Discussion

To determine the highest busulfan dose that NOD/SCID mice can tolerate, mice were treated with increasing doses of Busilvex[18] and survival was evaluated. At a dose of 35 mg/kg, all mice (5/5) lived up to 6 weeks, while at doses of 50 and 75 mg/kg, all mice (10/10) died within a few minutes or hours following treatment. This acute toxicity was not due to busulfan itself but to dimethyl acetamide, the adjuvant present in the solution. In order to reduce this immediate toxicity while at the same time maximizing myelosuppression, further experiments were performed with either a single dose of 35 mg/kg delivered 36 hours prior to transplantation, or two doses of 22 or 25 mg/kg delivered 48 and 24 hours prior to cell injection. We assessed human HSC engraftment kinetics following transplantation of CD34-purified cord blood cells into busulfan-treated mice and irradiated controls. The degree of human chimerism in the murine BM was determined by flow cytometry at 2.5 and 6 weeks in aspirated BM, and at sacrifice after 12 weeks in all other bones (Figure 1A-G). After 2.5 weeks, mice conditioned with 35 mg/kg of busulfan had a significantly smaller human graft compared to that of irradiated mice (Figure 1H; p<0.05). Subsequently, however, mice in both groups had similar grafts in both the samples of aspirated femur (6- and 12-week time points) and in the other pooled bones (analyzed at 12 weeks). Mouse survival rate at 12 weeks was similar for both conditions (25 of 30 busulfan-injected mice and 24 of 30 irradiated mice surviving). Mice receiving two injections of 22 mg/kg busulfan exhibited similar engraftment kinetics compared to those treated with a single dose of 35 mg/kg, but had greatly improved survival (29 of 30 at 12 weeks). In mice receiving two doses of 25 mg/kg busulfan, the level of engraftment was comparable to the level observed in irradiated mice at 2.5 weeks, but dramatically higher at 6 weeks (p<0.01), with equivalent survival (24 of 29). At 12 weeks, the degree of chimerism in both the aspirated femur and pooled bones from all groups of mice was similar (Figure 1H), however, there was a trend towards larger human grafts in mice treated with 25 mg/kg (Figure 1I). We performed a more detailed analysis of the composition of the human graft in recipient mice. At 2.5 weeks, mice in all groups had a high proportion of $CD45^-$ erythroid cells ($CD36^+$ and $CD36^-GPA^+$) in the BM, with a small $CD45^+$ population comprised mostly of $CD45^+CD36^+$ monocytic cells (Figure 1B and J). Early myeloerythroid repopulation by so-called rapid SRC[9,19] or short-term repopulating cells[9,20] has previously been described following intra-osseous injection of NOD/SCID mice or intravenous transplantation of NK-depleted NOD/SCID mice. In the current study, the percentage of $CD45^+$ cells in the graft was equivalent in all conditions; however, we observed differences in overall engraftment levels due to a higher percentage of erythroid cells in irradiated mice compared to in mice treated with 35 mg/kg or two doses of 22 mg/kg busulfan (Figures 1H and J; p<0.01). This may be related to increased sensitivity of short-term repopulating cells to residual non-dividing mature cells such as NK cells and monocyte-macrophages that are not fully depleted by the anti-CD122 treatment, and which are not eliminated as efficiently by busulfan as by irradiation. By 6 weeks, the erythroid population had decreased dramatically (Figures 1E and J). At this time point, the graft was predominantly $CD45^+$ (Figures 1E and J), with mainly $CD19^+$ lymphoid B cells and a minor proportion of $CD33^+$ myeloid cells (Figures 1G and K). There were no significant differences in the composition of the human graft among mice from any of the treatment groups. These data show that conditioning with busulfan or irradiation does not affect the long-term differentiation ability of repopulating HSC.

In order to compare the efficacy of stem cell homing under different conditioning regimens, we quantified the frequency of SRC by limiting dilution analysis. A range of doses of CD34-purified cells was injected into mice treated with either irradiation or two doses of 25 mg/kg busulfan. As shown in Table 1, the degree of chimerism analyzed at 6-7 weeks was proportional to the number of injected cells in both groups of mice, with higher levels of engraftment seen in busulfan-treated mice, in agreement with the data shown in Figures 1H and J. However, there was no significant difference in the calculated frequency of SRC detected

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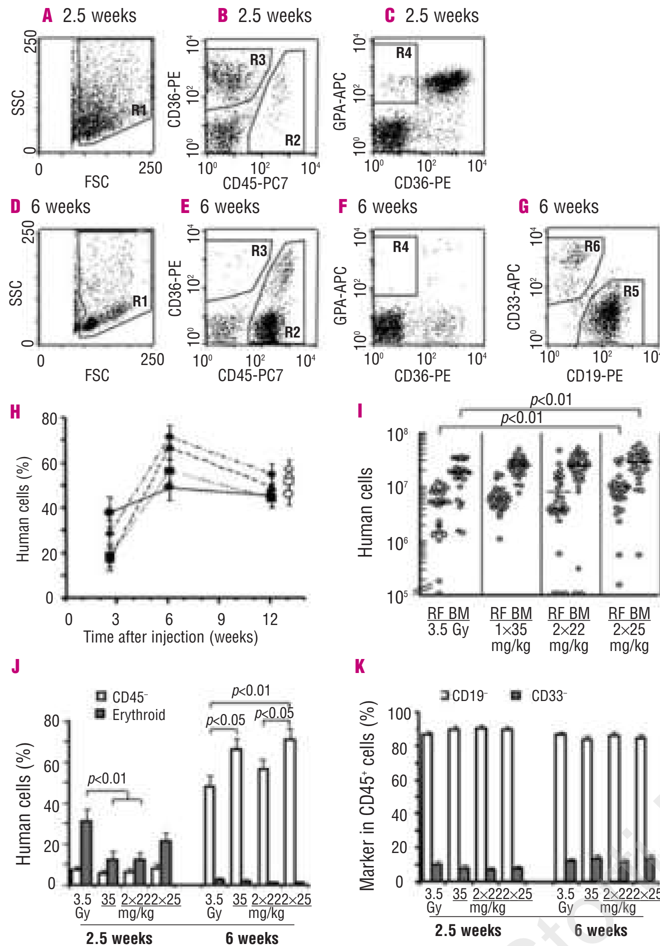

Figure 1. Human cell engraftment after irradiation and busulfan conditioning in NOD/SCID mice. A-G. Flow cytometry analysis of the human graft in a representative irradiated mouse. BM cells harvested from the aspirated right femur (RF) were examined by flow cytometry for the presence of nucleated viable cells at 2.5 weeks (A, region R1) and 6 weeks (D, R1). Region R1 was then used for the assessment of human cells composed of myelo-lymphoid CD45$^+$ cells (B and E, R2) and both CD45$^-$CD36$^+$ (B and E, R3) and CD36$^-$GPA$^+$ erythroid cells (C and F, R4). The percentage of CD45$^+$ cells expressing the CD19$^+$ lymphoid (G, R5) and the CD33$^+$ myeloid (G, R6) markers was determined by gating on the region R2. H. Human graft kinetics. CD34$^+$ cells (7.1±0.9x10^4) were intravenously injected into 109 mice (5 experiments). The level of total (erythroid and non-erythroid) human cell engraftment was assessed in the aspirated RF (closed symbols) at weeks 2.5, 6 and 12, and in the BM of non-aspirated pooled bones (contralateral femur, tibias and pelvis) at sacrifice after 12 weeks (open symbols). Recipients were treated by either irradiation (3.5 Gy, circles) or busulfan at 35 mg/kg (triangles), 2×22 mg/kg (squares) or 2×25 mg/kg (diamonds). I. Human cell quantification. Absolute number of human cells harvested from the RF and pooled bones (BM) after 12 weeks. J and K. Multilineage differentiation. Lineage analysis of the human graft in the murine BM by flow cytometry. J, non-erythroid (CD45$^+$) and erythroid (CD45$^-$CD36$^+$ and CD45$^-$CD36$^-$GPA$^+$) cell fractions at the 2.5- and 6-week time points. K, the proportion of B-lymphoid (CD19$^+$) and myeloid (CD33$^+$) cells in the CD45$^+$ population at 6 and 12 weeks. Data are presented as the mean ± SEM in H, J and K.

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in irradiated or busulfan-treated mice (1 SRC in 2,752 injected cells and 1 in 2,051, respectively). To rigorously test for primitive, self-renewing stem cells, we performed secondary transplantation experiments. At 12 weeks, total BM from primary mice was analyzed for the presence of human cells and then injected into secondary irradiated recipients. The intra-osseous technique was used for secondary transplantation in order to achieve better levels of engraftment and to enable assessment of ability to migrate from the injected femur to other bones, a property of stem cells.[9,19,21,22] In both primary and secondary mice, there were no differences between the two treatment groups in the degree of human chimerism in all hematopoietic tissues analyzed (Figure 2A). The number of HSC engrafting the BM, as demonstrated by colony-forming cell content, was also identical in irradiated and busulfan-treated mice after primary and secondary transplantation (Figure 2B). The observation that similar numbers of stem cells were able to migrate from the injected femur to non-injected bones confirms that irradiation and busulfan treatment have similar

Table 1. SRC frequency determined by limiting dilution assay in NOD/SCID mice treated by either irradiation at 3.5 Gy or 2 x 25 mg/kg busulfan.

Cell doses	*Irradiation (3.5 Gy)*			*Busulfan (2 x 25 mg/kg)*		
	Human cells (%)	*Positive mice per total injected mice*	*Positive mice (%)*	*Human cells (%)*	*Positive mice per total injected mice*	*Positive mice (%)*
330	< 0.1	1/12	8.3	< 0.1	2/12	17
1 000	< 0.1	2/9	22	1.4±1.1	4/11	36
3 300	2.6 ± 0.8	18/23	78	4.8±1.8	19/21	90
10 000	9.5 ± 2.0	16/17	94	24.3±4.9 *	14/15	93
> 30 000	45.9 ± 6.0	16/16	100	56.7±4.9	13/13	100
SRC frequency		1/2752			1/2051	
range		1/2248-1/3368			1/1659-1/2535	

*Results are from six independent experiments. *p<0.01 vs irradiation.*

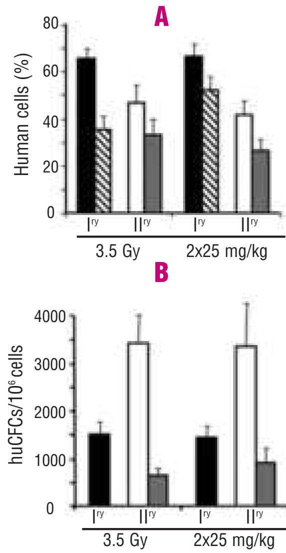

Figure 2. Human engraftment in primary and secondary mice. Human cells were analyzed in primary mice (Iry; BM, black bars; spleen, striped bars; n=33 mice from three experiments) 12 weeks after intravenous injection of $1.0\pm0.2\times10^5$ $CD34^+$ cells, and in secondary mice (IIry; BM of injected femur, white bars; non-injected femur and tibias, gray bars) 7 weeks after intraosseous injection of BM cells from primary mice. (A) Flow cytometry determination of the degree of human chimerism. (B) Estimation of human colony-forming cells (huCFC) content in the murine BM. Error bars represent SEM.

conditioning efficacy. The development of a chemotherapeutic conditioning protocol for the NOD/SCID mouse assay, which is comparable to the standard irradiation protocol, will certainly be of great help to laboratories that have limited access to a source of radiation. We chose to study busulfan with its myeloablative properties, rather than a highly immunosuppressive agent such as cyclophosphamide, because NOD/SCID mice already have a profound immunodeficiency. Previous mouse studies of busulfan conditioning used busulfan that required solubilization in dimethylsulfoxyde, a step that could affect the concentration and activity of busulfan, and consequently, the reproducibility of experiments. In humans, oral formulations are frequently used resulting in wide variations in plasma concentrations between patients. In this work, we took advantage of the clinical development of injectable forms of busulfan to investigate chemical conditioning of NOD/SCID mice prior to transplantation of human cells. We have established a reproducible modified NOD/SCID xenotransplantation system for detection of SRC that is equivalent to the standard assay using irradiation as the conditioning regimen. This modification will allow many laboratories that do not have access to irradiators to perform *in vivo* assays for human HSC, facilitating their input into the field of stem cell biology.

ER-R: PhD student who perform the experiment; CG: technical assistance for analysis; JO: technical assistance for irradiation; XS: technical assistance for antibody purification; IL-G: technical assistance for stem cell purification; HdV: director of the INSERM E0217 laboratory, revising the manuscript critically for important intellectual content; FM: research associate, leader of the project, expert in stem cell transplantation in mice.

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