Observations on inhibition of plaque-formation by spleen cells of antigenically stimulated animals on addition of theophylline in vitro[27] are not comparable with the results described above, where the influence of S8CT on sensitization in vivo was tested. The effect of S8CT on immune processes may not only be due to inhibition of cAMP degradation and therefore to a higher intracellular cAMP level because - under the same experimental conditions - antibody formation is described to be inhibited by cAMP[28] and on the other hand to be stimulated by theophylline[3,10,11].

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Viability of rabbit bone marrow after cryopreservation (in vitro and in vivo)[1]

E. Kühnlein, S. Casanova, A. Gratwohl[2], H. Haak and B. Speck

Division of Hematology, Department of Internal Medicine, Kantonsspital, CH-4031 Basel (Switzerland), 6 August 1980

Summary. A technique for preservation of rabbit bone marrow is described, which preserves viability of stem cells in all 22 animals as tested by autologous bone marrow transplantation and in vitro growth. Erythroid precursors survived better than myeloid precursors as observed by in vitro and in vivo recovery.

Combined antimitotic chemotherapy and high dose irradiation are used for successful treatment of patients with disseminated malignancies[3,4]. These therapies cause a dose-dependent bone marrow aplasia. The period of aplasia can be successfully shortened by an infusion of autologous bone marrow, aspirated before the beginning of therapy and preserved by deep freezing[5,6]. We modified the technique used by Appelbaum and Gorin[6,7] in canine experiments and in human marrow transplantation so that it could be applied in a rabbit model, and we tested the viability of the cryopreserved bone marrow by measuring its ability to repopulate the autologous bone marrow after 1200 rad total body irradiation and by observing in vitro growth of erythroid (BFU_E) and myeloid (CFU_C) precursors.

Materials and methods. In vivo. 1. Animals. Normal outbred New Zealand rabbits weighing 2.5–3.5 kg were used.

2. Bone marrow aspiration. Rabbits were anesthetized with Hypnorm (Philips-Duphar, B.V.). Bone marrow was aspirated from both iliac crests and femurs and put in sterile tubes containing 5 ml TC 199 (tissue culture medium, DIFCO) and 1000 IU heparine (Liquemin®, Roche). After aspiration the bone marrow was filtered and spun at 1000 rpm for 20 min (Hettich Rotanta centrifuge). The fat and plasma were removed and the nucleated cells were counted (Coulter Counter S). No attempt was made to separate the erythrocytes. TC 199 with 20% DMSO (Dimethylsulfoxide, BDH) and 10% autologous plasma was used as cryoprotective agent[8].

3. Freezing. The whole bone marrow volume was devided into 4 portions and transferred into UCAR blood freezing bags. Immediately after adding an equal volume of the cryoprotective agent (resulting in a final concentration of 10% DMSO), the bone marrow was frozen at a rate of 1 °C/min in a biological freezing apparatus (LINDE BF-4, Union Carbide corp.). The frozen material was stored at − 140 °C in the vapour phase of liquid nitrogen for 5–32 days.

4. Total body irradiation. 1 day before transplantation, the rabbits were exposed to a midline tissue dose of 1200 rad total body irradiation (Co^{60} Gammatron) at a rate of 35 rad/min.

5. Bone marrow transplantation. The 4 bone marrow portions of each individual animal were transplanted on the 2 following days. They were thawed quickly in a water bath at 37 °C. The rabbits were anaesthetized with Hypnorm® (Philips-Duphar, B.V.). After an injection of 0.5 ml Sandosten-Ca® (Sandoz) i.v., the thawed bone marrow was slowly

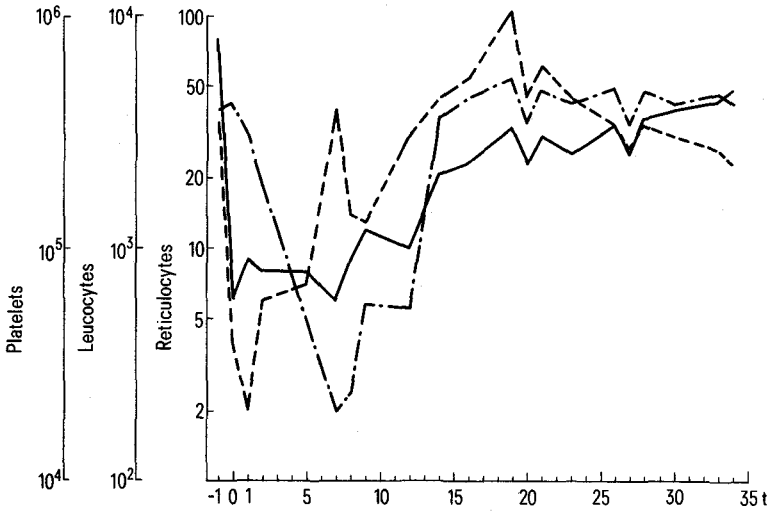

Fig. 1. Peripheral blood values (median) for 10 rabbits following total body irradiation and autologous bone marrow transplantation. Platelets, -·-·-·-; leucocytes, ——; reticulocytes, ----. Day −1 = Day of total body irradiation.

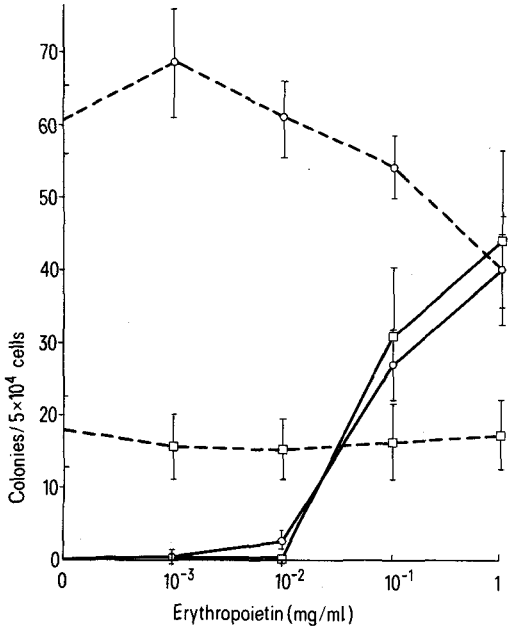

Fig. 2. Number of erythroid (BFU_E) and myeloid (CFU_C) colonies before and after cryopreservation at increasing doses of erythropoietin. Symbols represent means and SEM for 12 animals. BFU_E before cryopreservation, O—O; BFU_E after cryopreservation. □—□; CFU_C before cryopreservation. O---O; CFU_C after cryopreservation, □---□.

Transplantation of autologous cryopreserved bone marrow

Sex	Weight day 0	Aspirated b.m. (ml)	Storage time (days)	Number of cryopreserved cells/kg	Survival time (days)
f	3100	40	9	1.35×10^8	35
m	3150	42	8	1.14×10^8	*
m	3350	45	7	1.13×10^8	35
m	3000	30	6	0.80×10^8	35
f	3000	32	5	1.25×10^8	35
f	3700	38	32	0.70×10^8	35
f	3150	35	24	1.22×10^8	35
m	3150	39	29	1.34×10^8	35
f	3500	35	28	1.03×10^8	35
f	3300	36	28	0.64×10^8	35

f, female; m, male.

(maximum 1 ml/min) infused into the main vein of one ear without any prior separation procedures. The 2 infusions on the same day were given about 4 h apart.

6. Supportive care. During 35 days weight, excrements and clinical condition were controlled daily and complete blood counts were made 3 times a week. As long as the rabbits were pancytopenic they were given 1 ml Bactrim® (Roche) p.o. in order to prevent an infection.

In vitro. 1. Bone marrow. Bone marrow was aspirated as described. About 4 ml of bone marrow plus cryoprotective medium were used before and after freezing.

2. Preparation. The bone marrow solution was filtered to avoid clumps and then diluted 1:3 with Dulbecco's medium (GIBCO). 5 ml of this suspension were layered on to 4 ml of Ficoll-Ronpacon (d = 1.083) in Falcon tubes and spun for 25 min at 2000 rpm. The mononuclear cells were collected and washed in Dulbecco's medium. The thawed samples were handled similarly, except that DNA-ase was added after thawing.

3. Cultures. The cultures contained 5×10^4 cells in 1 ml IMDM (Iscove's modified Dulbecco's medium, GIBCO) with supplement agents and increasing doses of erythropoietin (human DEAE purified urinary erythropoietin) and HLCM (human leucocyte conditioning medium).

Results. In vivo. The results are summarized in the table and in figure 1. A mean number of 3.8×10^8 cells were aspirated in a mean volume of 37 ml, amounting to a mean value of 1.1×10^8 cells/kg b.wt. 3-4 bags of bone marrow were given. In all cases a recovery of the peripheral blood values were observed. All animals were killed at day 35. 1 animal, killed at day 8 because of a broken neck, showed a cellular bone marrow at post mortem examination. Reticulocytes began to increase after the post irradiation nadir in all animals before leucocytes and thrombocytes began to increase (figure 1).

In vitro. Results are summarized in figure 2. In all cultures, stem cells had survived and formed colonies. Erythroid colonies (BFU_E) grew as well after thawing as before and showed the same growth dependence on erythropoietin. Growth of myeloid colonies (CFU_C) was significantly suppressed after cryopreservation. In three out of twelve animals no CFU_C's grew, despite the fact that a normal growth of BFU_E's was seen with the same marrow.

Discussion. Bone marrow can successfully be cryopreserved and be used to prevent prolonged aplasia[5-7,9]. Our study shows that the same methods used successfully in man and in dogs can be applied in a rabbit model. All rabbits had a repopulation of bone marrow, whereas only 2 of 14 animals

treated with total body irradiation alone survived and recovered (unpublished data). However some precautions are necessary. In preliminary studies we saw that an infusion of more than 10 ml leads to acute right heart failure in the rabbit. If the total amount is split into single portions not containing more than 10 ml of volume, no such side effect is seen. A maximum number of cells is desirable, since the duration of aplasia is directly related to the number of bone marrow cells reinfused[6]. This was the reason that in vitro and in vivo studies were performed in separate animals. Our study also shows that with this freezing technique, which is commonly used in clinical studies[5], not all hematopoietic precursors are ideally preserved. The aim of our study was not to find the optimal freezing technique for rabbit bone marrow but to test an established technique for its use in the rabbit. Using this technique erythroid precursors survive better. They grow in vitro as well as before cryopreservation and reticulocytes recover first in vivo (figures 1 and 2). Growth of myeloid colonies, however, is clearly impaired in vitro. The fact that others have described normal recovery of CFU_C after cryopreservation is not surprising[9]; they adjusted their technique for optimal recovery of CFU_C but did not control the recovery of erythroid precursors. This clearly shows that assessing just one function can lead to erroneous results and does not necessarily represent total bone marrow function. In conclusion we can say that the rabbit is a useful model for assessing the use of cryopreservation of bone marrow and that with the established technique the bone marrow will repopulate the empty autologous bone marrow and will grow in vitro. Further studies are needed to evaluate how erythroid and myeloid precursors can both be optimally preserved.

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Effects of retinoic acid on the fibrinolytic activity of HL 60 human promyelocytic leukemia cells

F. Ghezzo and L. Pegoraro

Clinica Medica I, Istituto di Medicina Interna, Università di Torino, Corso Polonia 14, I-10126 Torino (Italy), 25 July 1980

Summary. Fibrinolytic activity of HL 60 human leukemic cells was found to increase in parallel with myeloid differentiation following retinoic acid but not dimethylsulfoxide treatment. However, both retinoic acid and dimethylsulfoxide produced an increase in acid phosphatase and a decrease in muramidase.

Retinoic acid (all-trans-retinoic acid) and retinol are essential factors for the normal differentiation of epithelial cells[1]. They also seem to be active in preventing chemical induction and development of epithelial tumors as well as in promoting in vitro and in vivo differentiation of some neoplastic epithelial cells[2-6]. The ability of retinoic acid to promote differentiation does not seem to be limited to epithelial cells, since myeloid leukemic cells of murine origin are also induced to differntiate in vitro, exhibiting an increased lysosomal enzyme activity[7]. More recently Breitman et al.[8] have demonstrated that the treatment of a human promyelocytic leukemia cell line (HL 60) with retinoic acid increases the percentage of cells with the ability to reduce nitroblue tetrazolium salts (NBT), a marker of myeloid differentiation. In the present study we report the effects of retinoic acid on the fibrinolytic activity of HL 60 cells. Since cellular fibrinolytic activity is dependent on lysosomal enzymes, acid phosphatase and muramidase, 2 markers of lysosomal activity, were also studied.

Materials and methods. HL 60 cells (a gift from Dr Rovera, Wistar Institute) were grown in RPMI 1640 supplemented with 12% fetal calf serum (FCS) in 5% CO_2 incubator. Retinoic acid (Sigma, USA) was dissolved in 98% ethanol and used at the final concentration of 1×10^{-6} M; dimethylsulfoxide (DMSO) Sigma, USA) at the final concentration of 1.2%. NBT (Sigma, USA) reduction was assayed as described by Collins et al.[9]. The percentage of differentiated cells was determined on cytocentrifuge slides stained with May Grünwald-Giemsa. Fibrinolytic activity was assayed by the ^{125}I-fibrin plate method of Unkeless et al.[10]: fibrinogen (Kabi, Sweden) was dissolved in water at the concentration of 100 µg/ml and mixed with ^{125}I-fibrinogen (Sorin, Italy) to obtain 60,000 cpm/ml, and placed in 35 mm diameter plastic dishes at the concentration of 10 µg/cm^2. Fibrin was obtained by incubating the plates at 37 °C for 2 h with acid-treated 5% FCS. 2×10^5 cells were resuspended in 1 ml of 0.15 M tris buffer pH 7.8, plated on fibrin plates and incubated for 4 h at 37 °C. Fibrinolysis was evaluated by measuring the radioactivity of the supernatant in a γ-counter. Addition of retinoic acid and DMSO at the same concentrations used in the cellular cultures to the fibrin plates did not produce lysis of fibrin nor affected exogenous urokinase fibrinolytic activity. Plasminogen-free fibrin plates obtained by the method of Lassen[11] allowed us to evaluate the proportion of non plasminogen activation mediated fibrinolytic activity, which was found to be about 50% of the total activity. For the determination of acid

Fibrinolysis, acid phosphatase and muramidase activities of HL 60 cells untreated and following a 4-day treatment with retinoic acid and DMSO

	Fibrinolytic activity*	Acid phosphatase**	Muramidase***
HL 60	1.17 ± 0.2503	107 ± 9.90	3.90 ± 0.1414
HL 60 + retinoic acid	3.51 ± 0.2691	255 ± 7.07	1.75 ± 0.2121
HL 60 + DMSO	0.65 ± 0.1813	188 ± 8.48	1.52 ± 0.1061

* µg of fibrin digested in 4 h at 37 °C/plate; ** pM of p-nitrophenyl phosphate hydrolyzed/min/1×10^6 cells; *** ng of egg white muramidase/1×10^6 cells.