Case reports

Red Blood Cells (RBC) and High Fluorescence Reticulocytes (HFR) production increased by induction chemotherapy and GM-CSF plus G-CSF in peripheral blood of breast cancer patients

S. Charrier[1], G. Portefaix[2], P. Chollet[2], F. Kwiatkowski[2], Y. Communal[2], H. Curé[2], J.-O. Bay[2], G. Bétail[1], R. Plagne[2] and J. Chassagne[2]

[1] Faculté de Médecine et Pharmacie, 28 place Henri Dunant, BP 38, F-63001 Clermont-Ferrand, France

[2] Centre de lutte contre le cancer Jean Perrin, 58 rue Montalembert, BP 392, F-63011 Clermont-Ferrand, France

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Correspondence to: S. Charrier, Inserm U362, Hématopoïèse et Cellules Souches, Institut Gustave Roussy PR-1, 39/53, Rue Camille Desmoulins, 94805 Villejuif Cedex, France, E-mail address : scharrier@igr.fr

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Abstract

Abstract. The preservation of erythrocytes is important during cytostatic chemotherapy. To study the synergistic effect of GM-CSF plus G-CSF on the red blood recovery, we looked at the kinetics of red blood cells, total reticulocytes and High Fluorescence Reticulocytes (HFR) from peripheral blood of 26 breast cancer patients, between 2 cycles of chemotherapy. After four days of TNCF (Thp-doxorubicin, Vinorelbine, Cyclophosphamide, Fluorouracil) treatment, 12 patients received either GM-CSF or G-CSF (D5-D16) and 14 received both of them (GM-CSF, D5-D14 and G-CSF, D10-D14). Our results showed a significantly lesser RBC decrease for patients receiving both CSFs versus only one CSF ($p < 0.05$). This greater RBC preservation was explained by the increase of HFR and total reticulocytes from D12 to D17 in the peripheral blood of patients receiving the two CSFs, with significantly values greater than that of patients receiving one CSF ($p < 0.01$) it showed that the synergistic GM-CSFplus G-CSF effect observed previously on PBPC results in a positive effect on erythroid lineage. GM-CSF plus G-CSF association exhibit *in vivo* multilineage (myeloid and erythroid) activity showing the interest to use different cytokines in combination to obtain a better hematological recovery after induction chemotherapy as well as a better quality of PBPC mobilization for transplantation after high-dose chemotherapy.

The regulation, the proliferation and the differentiation of hematopoietic progenitors *in vitro* are dependant on cellular hematopoietic growth factors, also known as CSFs, colony-stimulating factors [1]. These cytokines are usually classified by the types of mature cells found in the colonies to which they give rise in response to the differentiation process they stimulate. The isolation of several CSFs allowed to understand better the hematopoiesis pattern and to found them rapidly a clinical use in medical oncology [2-6].

In this therapeutic approach, two main factors [7] are used granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF). GM-CSF and G-CSF are produced, *in vivo*, by endothelial cells and are known to be involved in the regulation, growth and differentiation of hematopoiesis [8]. GM-CSF is a regulator of the intermediate stages of hematopoiesis, it supports the expansion and growth of both granulocytic and monocytic colony forming units (CFU-GM). G-CSF, a later-acting cytokine, is a lineage-specific growth factor that regulates the production and function of neutrophils. As *in vitro* studies

have shown that GM-CSF and G-CSF have different biological and chronological actions [9], and that the combination of both seemed to have a synergistic interaction [10-12], it appeared us interesting to see their *in vivo* action after chemotherapy and GM-CSF plus G-CSF combination. In a previous study, from 43 breast cancer patients, we studied the hematological recovery and PBPC mobilization after chemotherapy and GM-CSF plus G-CSF versus G-CSF or GM-CSF alone [13]. Our results showed the synergistic effect of these two cytokines in combination for PBPC mobilization (CD34+, CFU-GM, cells in cycle). For hematological recovery, leukocyte and mononuclear cell counts were significantly higher for patients receiving GM-CSF plus G-CSF or G-CSF alone than those receiving GM-CSF alone ($p < 0.001$).

However, as some *in vitro* [14, 15] and *in vivo* [16, 17] studies have shown an effect of GM-CSF or G-CSF on erythropoiesis lineage, it seemed us interesting to study the CSFs combination versus one CSF (GM-CSF or G-CSF) on erythropoiesis. The reticulocyte count is an important parameter to evaluate the degree of effective erythropoiesis [18]. Advances in automated by flow cytometry have led to the development of more exact counting of reticulocytes, based on reticulocytes ARN staining with thiazole orange, providing an objective measure of their maturity in an easy, reliable and reproducible method [19]. These counters are able to classify reticulocytes into three different stages of maturation, according to the level of ARN the more mature reticulocytes with a low fluorescence (LFR), the medium fluorescence reticulocytes (MFR) and the more immature reticulocytes with high fluorescence (HFR) [20]. These new parameters have generated clinical interest in the study of anemias [21] and in monitoring post-therapeutic hematological recovery [22-24].

Therefore, to know the effect of GM-CSF and G-CSF combination on the erythropoiesis, we performed here a study looking at the kinetics of red blood cells, total reticulocytes and HFR from peripheral blood of breast cancer patients receiving induction chemotherapy prior CSF support (G-CSF alone or GM-CSF alone or GM-CSF plus G-CSF combination). Considering the excellent efficacy of the TNCF (Thp-doxorubicin, Vinorelbine,Cyclophosphamide, Fluorouracil) regimen combining at the same time both mobilizing [25] and specific cytotoxic effects [26], we have chosen the breast cancer patients treated by this chemotherapy applied at the maximally tolerated dose with CSF support.

In this purpose, in 26 patients treated by TNCF regimen, red blood cells were analyzed between two cycles of chemotherapy and reticulocytes were studied for 6 days, beginning when leukocytes were greater than 1.010^{9}/l of blood.

Patients and methods

Between March 1996 and December 1997, 26 breast cancer patients with a poor prognosis were enrolled in this study. The criteria of eligibility for these patients was to have red blood cells count greater than $3.5\ 10^{12}$/l of blood before the start of chemotherapy. Patient characteristics are shown in Table 1. Nineteen had primary (neo)adjuvant and 7 had metastatic disease with a prior treatment in two cases. The median age of all patients was 43 years (range 33-59). Patients were treated by 3-week cycles of TNCF chemotherapy (Table 2) (D1 to D4) and received colony-stimulating factor support (G-CSF, GM-CSF or both of them), 5 µg/kg once daily, for an average of ten days (D5 to D16). Twelve patients received one CSF (7 GM-CSF and 5 G-CSF) and 14 patients, the combination of two CSFs (GM-CSF (D5-D14) plus G-CSF (D10-D14)).

CSF support	GM-CSF or G-CSF	GM-CSF plus G-CSF	Total
Evaluated Patients N°	12	14	26
Median age(range)	46 (33-59)	41 (33-53)	43 (33-59)
Breast cancerstage			
2a	/	2	2
2b	9	6	15
3a	/	1	1
3b	1	/	1
4	2	5	7
SBR grade			
1	2	3	5
2	5	3	8
3	3	6	9
TNCF treatment			
adjuvant	1	/	1
neoadjuvant	9	9	18
metastatic	2	5	7
Prior treatment	0	2	2

Table 1. Characteristics of breast cancer patients treated by TNCF chemotherapy

TNCF regimen	Drugs	Post-chemotherapy Days
	THP-Doxorubicin (20 mg/m²)	D1 to D3
	Vinorelbine (25 mg/m²)	D1 and D4
	Cyclophosphamide (300 mg/m²)	D1 to D4
	Fluorouracil (400 mg/m²)	D1 to D4
CSF support		
GM-CSF alone		D5 to D16
G-CSF alone		D5 to D16
GM-CSF + G-CSF		
GM-CSF		D5 to D14
G-CSF		D10 to D14

Table 2. Management ofTNCF treatment

Red blood cell (RBC) counts were performed before chemotherapy and daily from the start of aplasia until the next cycle. After the first or second TNCF cycle, from the day where white blood cells (WBC) were greater than 1.0 10^9/l of blood, total reticulocytes and HFR were evaluated daily. Median time of reticulocytes evaluation for each patient was 6 days from D12 until D17.

Reticulocytes staining. Blood samples were stained for flow cytometric reticulocyte analysis with 0.1 µg/ml of thiazole orange by a modification method of Lee et al. 1986 [19]. Fresh staining solution was made up daily by diluting a stock solution of 1 mg/ml of thiazole orange (Aldrich Chem Co, Milwaukee, WI) in ethanol 110,000 with phosphate-buffered saline (GIBCO Brl, Praisley, Scotland) at a pH of 7.4. Whole blood (5 µl) was added to 2 ml staining solution in 12 x 38-mm polypylene tubes and incubated in the dark for 30 to 60 minutes at room temperature with intermittent mixing. A similar incubation of a 5-µl sample of whole blood in phosphate-buffered saline was used as the unstained or autofluorescent control. Both samples were analyzed on flow cytometry.

Flow cytometry analysis was performed using an EPICS XL analyser (Coulter Corp, Miami, Fl). Fluorescence attributable to thiazole orange was determined using excitation by an argon laser operating at 488 nm. The acquisition gate included the entire red blood cell populations and excluded white blood cells. A minimum of 100,000 events was acquired in list mode for each sample.

Fluorescence histograms from the stained and unstained control samples were analyzed to calculate the percentage of total reticulocytes and HFR subpopulation. Circulating total reticulocytes and HFR cells per litter of blood was determined by multiplying their frequency with the red blood cell count in the same blood sample.

Expression of results and statistical analysis. Red blood cells, reticulocytes and HFR were expressed as an absolute count per litter of peripheral blood. For the figures, results were reported as the mean ± SEM and expressed according to the post-chemotherapy day. Kruskall-Wallis, Variance analysis (ANOVA) and matched pairs student tests evaluated differences between the mean values.

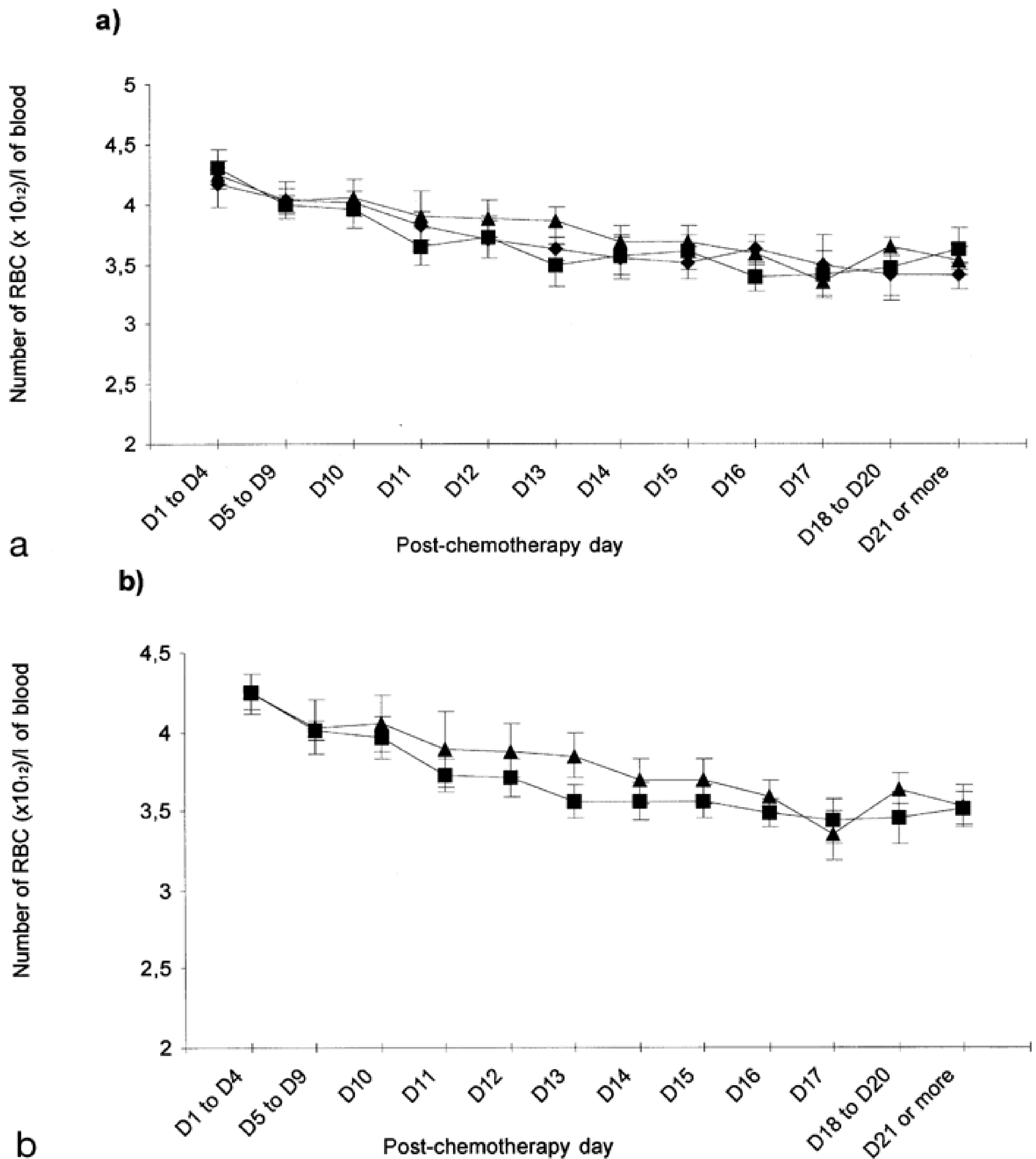
Results

Before to study the red blood recovery in breast cancer patients treated by chemotherapy and CSF, we evaluated total reticulocytes and HFR in 64 healthy donors 32 females and 32 males (Table 3). The mean values of total reticulocytes and HFR were similar in the two groups and we can consider that our normal mean values of total reticulocytes and HFR were respectively 67,868 (24,505-111,231) 10^6/l of blood and 2,582 (0-6,367) 10^6/l of blood.

		Male	Female	Total
Donor number		32	32	64
Total	Mean	66,953	68,783	67,868
reticulocytes	range	(27,300-131,760)	(27,860-118,750)	(27,300-131,760)
High flourescence	Mean	2,799	2,366	2,582
reticulocytes	range	(188-9,882)	(202-7,007)	(188-9,882)

Table 3. Normal value (10^6/l of blood) of total reticulocytes and high fluorescence reticulocytes from 64 healthy donors

Red blood cells.In this study, we evaluated 26 breast cancer patients (Fig. 1) 12 patients received GM-CSF (7 patients) or G-CSF (5 patients) alone and 14 patients GM-CSF plus G-CSF.



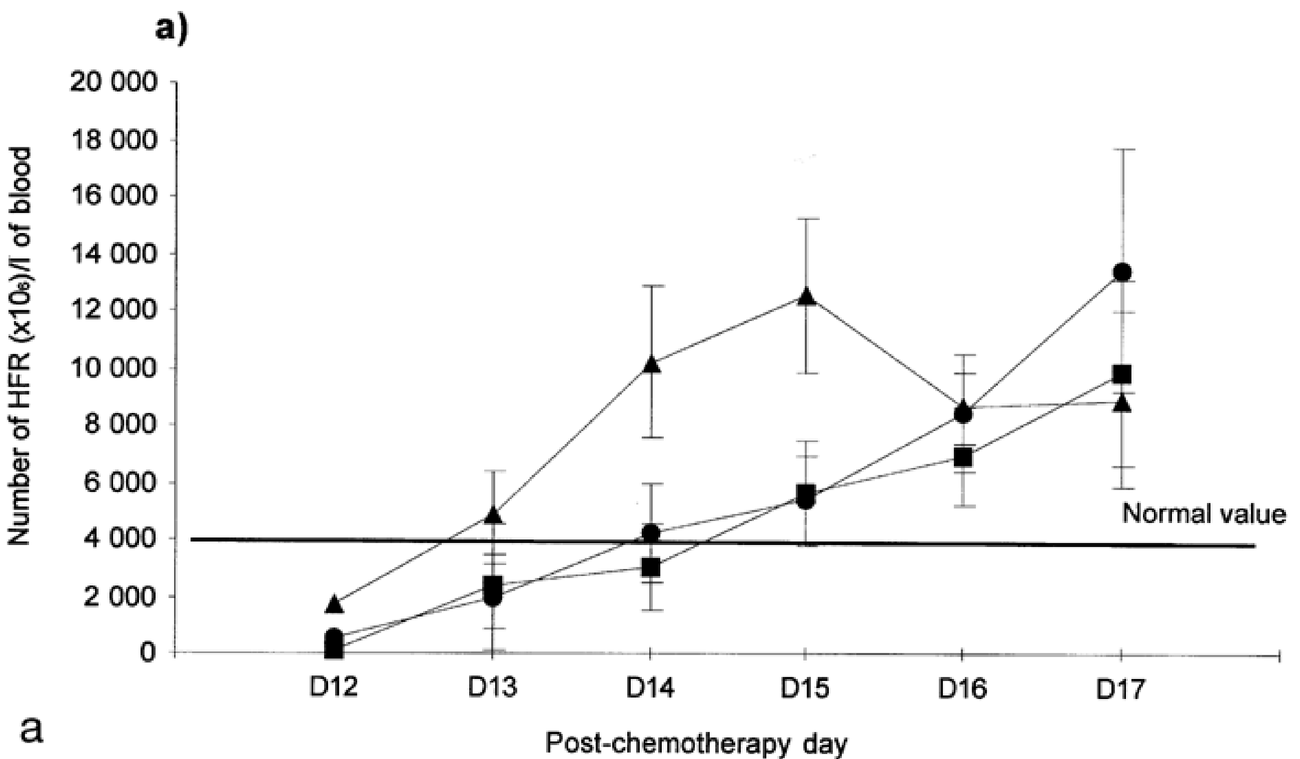
Figs. 1a, b. Hematological recovery (red blood cells) after TNCF cycle according to the post-chemotherapy day (mean ± standard error of the mean), (a) ♦ GM-CSF plus G-CSF, ■ GM-CSF and •;G-CSF, (b) ♦ GM-CSF plus G-CSF, ■ one CSF i.e. GM-CSF or G-CSF alone. (D1 to D4) is the start of TNCF cycle, (D14), the last day of G-CSF administration and (D21 or more), the next start of TNCF cycle

In the Figure 1a, the evolution of RBC counts between D1 and D21 was not significantly according to the CSF used (GM-CSF or G-CSF or the combination of GM-CSF plus G-CSF). However, if we separated the population only in two groups (patients receiving GM-CSF or G-CSF alone versus patients receiving GM-CSF plus G-CSF), we observed a significant difference between the patients receiving the combination of two

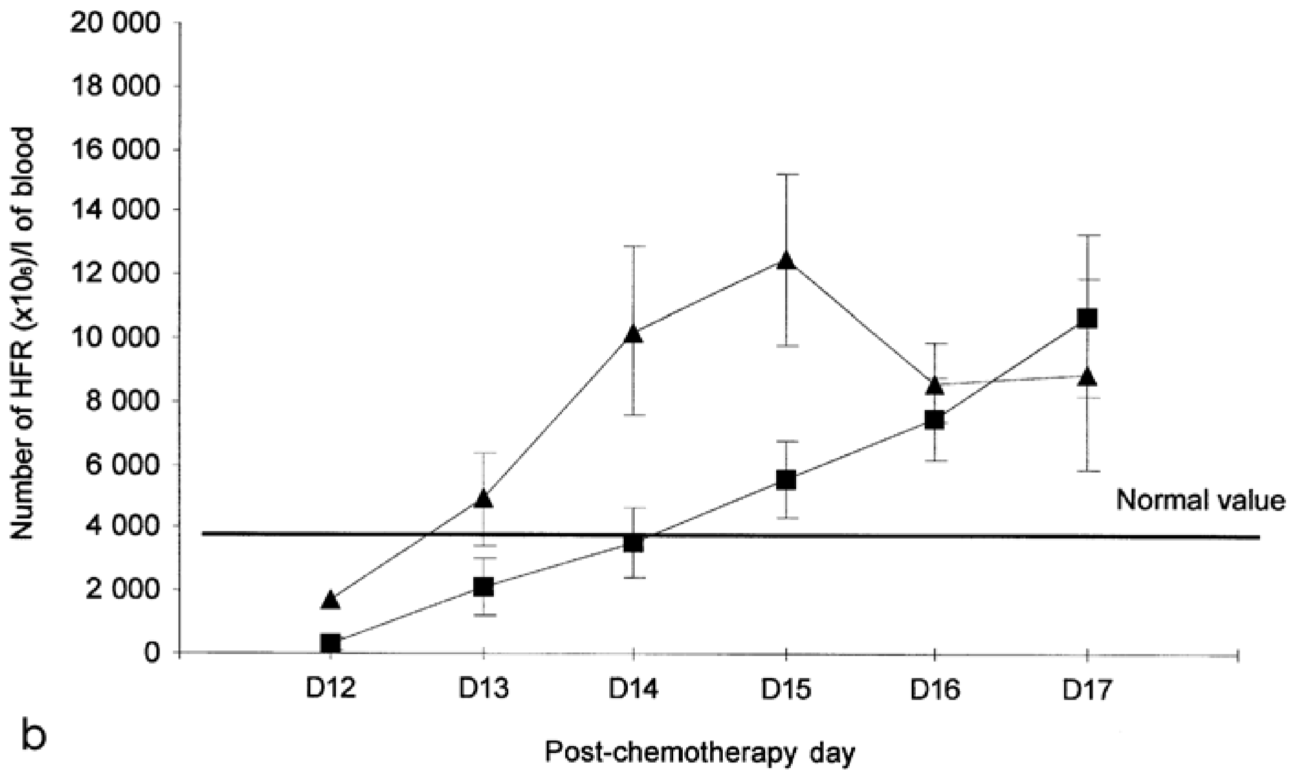
CSFs versus patients receiving one CSF ($p < 0.05$) (Fig. 1b).

On D1, the first day of chemotherapy administration, the mean red blood cells were 4.24 (3.52-4.90) 10^{12}/l of blood for patients receiving the combination of GM-CSF plus G-CSF and 4.25 (3.5-4.69) 10^{12}/l of blood for patients receiving one CSF. These values were found lower on D21 (or D1 of the next TNCF cycle) 3.53 (2.81-4.99) 10^{12}/l of blood for patients receiving GM-CSF plus G-CSF and 3.51 (3.09-3.91) 10^{12}/l of blood for patients receiving GM-CSF or G-CSF alone.

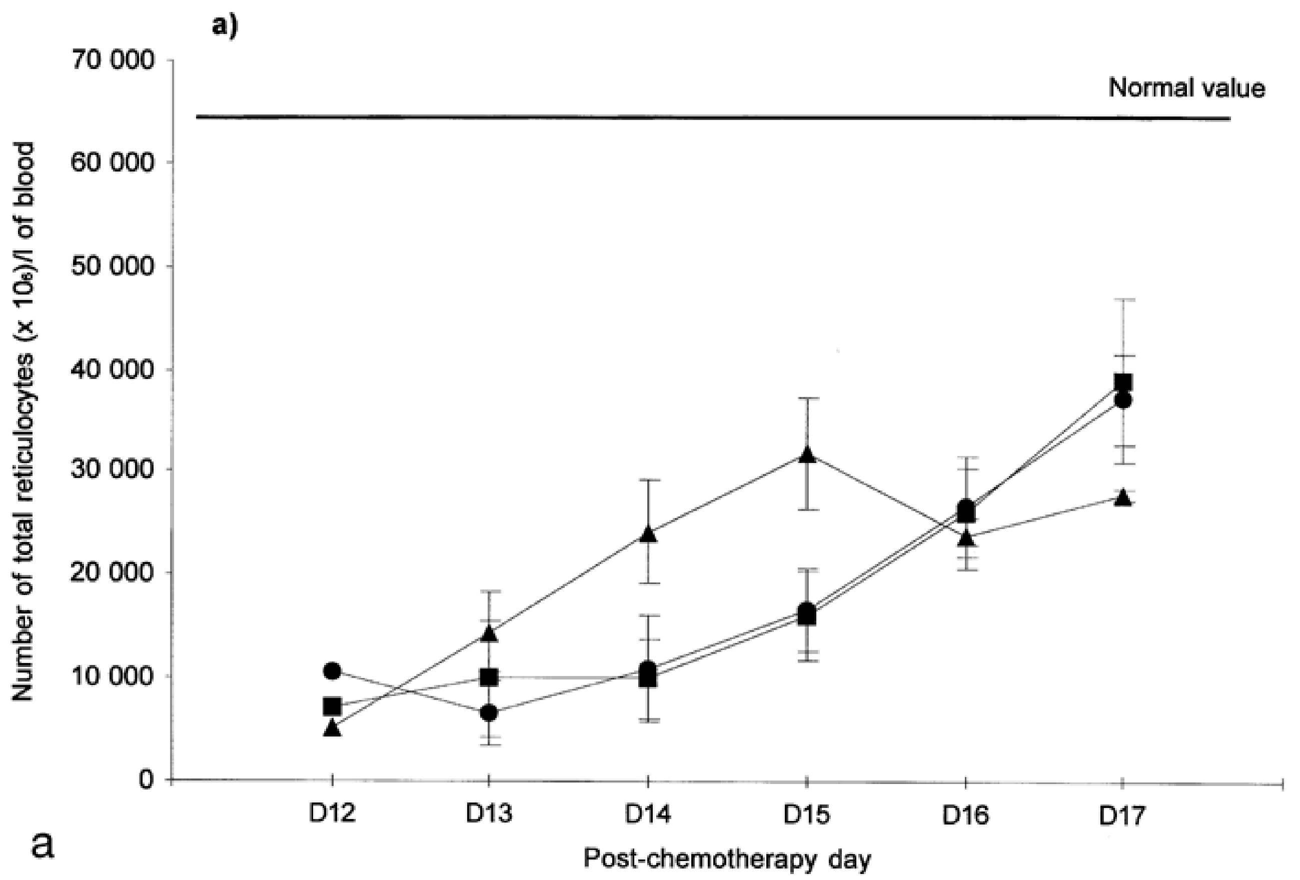
Kinetics of total reticulocytes (Fig. 2) and HFR (Fig. 3). The profile of these kinetics from D12 until D17 was significantly different between the two groups (GM-CSF plus G-CSF versus GM-CSF or G-CSF alone) ($p < 0.01$). For patients receiving one CSF, the values of total reticulocytes and HFR increased progressively reaching respectively on D17, 38,423 (11.162-59.445) 10^6/l of blood and 10.750 (3.049-22.733) 10^6/l of blood. Conversely, for the patients receiving the combination of two CSFs, the values of total reticulocytes and HFR were increased until D15, fitting to the optimal value 31.829 (8.845-79.833) 10^6/l of blood and 12.534 (5.075-35.637) 10^6/l of blood. As soon as the CSF was stopped, a progressive decrease in reticulocytes was observed until the end of the study.



b)



Figs. 2a, b. Total reticulocytes numeration between b the post-chemotherapy days D13 to D17 after TNCF cycle (mean ± standard error of the mean), (**a**) ♦ GM-CSF plus G-CSF, ■ GM-CSF and •; G-CSF, (**b**) s GM-CSF plus G-CSF, ■ one CSF i.e. GM-CSF or G-CSF alone



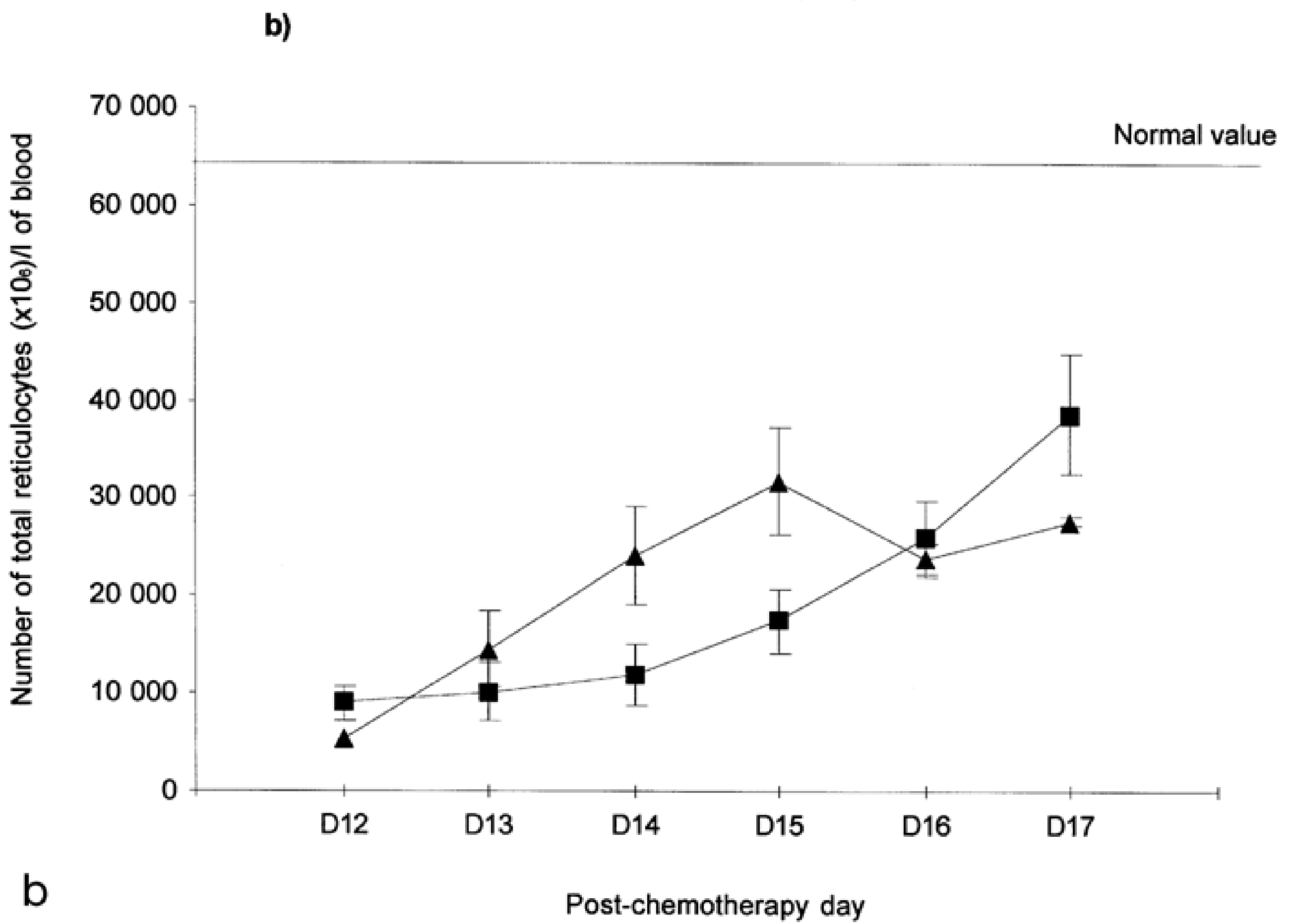


Fig. 3a, b. HFR numeration between the post-chemotherapy days D13 to D17 after TNCF cycle (mean ±

standard error of the mean), (**a**) s GM-CSF plus G-CSF, ■ GM-CSF and •; G-CSF, (**b**) ♦ GM-CSF plus G-CSF, n one CSF i.e. GM-CSF or G-CSF alone

The same phenomenon between the two groups (both CSFs versus one CSF) was observed for HFR and total reticulocyte kinetics but the values of total reticulocytes in Day 17 didn't reach the normal level.

Discussion

The CSFs are now well known to be involved in the regulation, growth and differentiation of hematopoiesis. Three hematopoietic growth factors are currently commercially available for clinical use GM-CSF, G-CSF and erythropoietin [8]. *In vitro* studies have shown that GM-CSF have an earlier chronological action than G-CSF [9] and that the combination of both CSF tend to have a synergistic interaction [10-12]. In a previous study, we have shown that the combination of GM-CSF and G-CSF could have a synergistic *in vivo* effect improving hematological recovery between two TNCF cycles and optimizing PBPC mobilization (CFU-GM, CD34+, cells in cycle) to allow only one leukapheresis. Indeed, the PBPC recruitment of patients receiving both CSFs was 2-fold greater than that of evaluated for patients receiving G-CSF, and 4-fold greater than that of patients receiving GM-CSF [13]. After to have seen the synergistic effect of the GM-CSF plus G-CSF combination on the myeloid lineage, we wanted to study if the same phenomenon was observed on erythropoiesis. Some *in vitro* studies have shown that GM-CSF or G-CSF added to others cytokines like erythropoietin could have a multilineage colony-stimulating activity [14, 15]. Consequently, we have evaluated here the regeneration of red blood cells, reticulocytes and HFR from peripheral blood of breast cancer patients receiving induction chemotherapy prior CSF support (G-CSF alone or GM-CSF alone or GM-CSF plus G-CSF combination).

In this present work, our results have shown a significantly better RBC recovery for the patients receiving GM-CSF plus G-CSF versus patients receiving GM-CSF or G-CSF alone. This better RBC kinetic can be explained by the increase of HFR and reticulocytes between D12 until D17 in the peripheral blood of patients receiving the two CSFs. These values were significantly greater than that of patients receiving one CSF, showing that the synergistic effect of GM-CSF plus G-CSF on PBPC could have a positive effect on erythroid lineage, by stimulating red blood precursors. Until now, we have found only two *in vivo* studies tending to show an effect of GM-CSF or G-CSF on erythropoiesis. The first study [16] showed an increase of reticulocytes after chemotherapy and G-CSF, but the action of G-CSF on erythropoiesis was not clearly demonstrated because in HFR fraction, no significant difference was found after day 2. In 1997, another study [17] was realized from Tg-mice transfected with GM-CSF receptors. Their results showed that the GM-CSF stimulated not only myelopoiesis but also erythropoisis and megakaryopoiesis of the GM-CSF receptor Tg-mice. In our study, when both CSFs were used, a synergistic effect dependent on CSF administration was observed with an increase of total reticulocytes, HFR and PBPC [13]. This supposes an increase of precursor cells, which would stimulate cytokines acting in self-renewal of the hematopoiesis (myelopoiesis and erythropoiesis) and an increase of endogenous cytokines like erythropoietin, dependent-cytokine of erythropoiesis [27].

In conclusion, in this present study, we see that the synergetic effect of GM-CSF plus G-CSF has an implication on multilineage and particularly, myeloid and erythroid lineages. As these cytokine have a lineage-specific, different and chronological action, it would appear more interesting to administrate *in vivo* different cytokines in combination to obtain a better hematological recovery after induction chemotherapy or a better quality of PBPC mobilization for transplantation after the high-dose chemotherapy.

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References

1. Burgess AW, Metcalf D (1980) The nature and action of granulocyte-macrophage colony stimulating factors. Blood 56 947-958

2. Gabrilove JL, Jakubowski A, Scher H, et al. (1988) Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transcripional-cell carcinoma of the urothelium. New Engl J Med 318 1414-1422

3. Hoekman K, Wagstaff J, Van Groeningen CJ, et al. (1991) Effects of recombinant human granulocyte-macrophage colony stimulating factor on myelosuppression induced by multiple cycles of high-dose chemotherapy in patients with advanced breast cancer. J Nat Cancer Inst 83 1546-1553

4. Trillet-Lenoir V, Green J, Manegold C, et al. (1993) Recombinant granulocyte colony stimulating factor reduces the infections complications of cytotoxic chemotherapy. Eur J Cancer 29A 319-324

5. Teshima T, Harada M, Takamatsu Y, et al. (1993) Granulocytecolony-stimulating factor (G-CSF) induced mobilization of circulating haemopoietic stem cells. Br J Haematol 84 570-573

6. Ayash LJ (1994) High-dose Chemotherapy with autologous stem cell support for the treatment of metastatic breast cancer. Cancer 74 532-535

7. American Society of Clinical Oncology recommendations for the use of hematopoietic colony-stimulating factors (1995) Evidence-based, clinical practice guidelines. J Clin Oncol 12 2471-2508

8. Vose JM, Armitage JO (1995) Clinical applications of hematopoietic growth factors. J Clin Oncol 13 1023-1035

9. Peters WP, Rosner G, Ross M, et al. (1993) Comparative effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocytecolony-stimulating factor (G-CSF) on priming peripheral blood progenitor cells for use with autologous bone marrow after high-dose chemotherapy. Blood 81 1709-1719

10. McNiece I, Andrews R, Stewart M, et al. (1989) Action of interleukin-3, G-CSF, and GM-CSF on highly enriched human hematopoietic progenitor cells Synergistic interaction of GM-CSF pluse G-CSF. Blood 74 110-114

11. Bot FJ, van Eijk L, Schipper P, et al. (1990) Synergistic effects between GM-CSF and G-CSF or M-CSF on highly enriched human marrow progenitor cells. Leukemia 4 325-328

12. Hogge DE, Cashman JD, Humphries RK, Eaves CJ (1991) Differential and synergistic effects of human granulocyte-macrophage colony-stimulating factor and human granulocyte colony-stimulating factor on hematopoiesis in human long-term marrow cultures. Blood 77 493-499

13. Charrier S, Chollet P, Bay JO, et al. (2000) Hematological recovery and peripheral blood progenitor cells mobilization after induction chemotherapy and GM-CSF plus G-CSF in breast cancer. Bone Marrow Transplant 25 705-710

14. Sieff CA, Emerson SG, Donahue RE, et al. (1985) Human recombinant granulocyte-macrophage colony-stimulating factor a multilineage hematopoietin. Science 230 1171-1173

15. Nishijima I, Nakahata T, Hirabayashi Y, et al. (1995) A human GM-CSF receptor expressed in transgenic mice stimulates proliferation and differentiation of hematopoietic progenitors to all lineages in response to human GM-CSF. Mol Biol Cell 6 497-508

16. Remacha AF, Martino R, Sureda A, et al. (1996) Changes in reticulocyte fractions during peripheral stem cell harvesting role inmonitoring stem cell collection. Bone Marrow Transplant 17 163-168

17. Nishijima I, Nakahata T, Watanabe S, et al. (1997) Hematopoietic and lymphopoietic responses in human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor transgenic mice injected with human GM-CSF. Blood 90 1031-1038

18. Hoewen B (1992) Reticulocyte maturation. Blood Cells 18 167-186

19. Lee LG, Chen CH, Chiu LA (1996) Thiazole orange a new dye for reticulocyte analysis. Cytometry 7 508-517

20. Hohenwallner W, Wiensenger K, Wimmer E (1992) The reticulocytes automatic counting, indication and interpretation. Sysmex Jint 2 120-135

21. Tsuda I, Tatsumi N (1989) Maturity of reticulocytes in various hematological disorders. Eur J Haematol 43 252-254

22. Tanke HJ, Rothbarth PH, Vossen JMJJ, et al. (1983) Flow cytometry of reticulocytes applied to clinical hematology. Blood 61 1091-1097

23. Aulesa C, Ortega JJ, Jou JM (1994) Flow cytometric reticulocyte quantification in the evaluation of hematologic recovery. Eur J of Haematol 53 293-297

24. Portefaix G, Communal Y, Fleury J, et al. (1996) Interest of high fluorescence reticulocytes (HFR) evaluation in optimal time determination of peripheral blood progenitor cell (PBPC) havesting. Blood 88 [suppl 10] 3721a

25. Charrier S, Chassagne J, Curé H, et al. (1998) Mobilization of peripheral blood progenitor cells after induction chemotherapy (THP-Doxorubicin-Vinorelbine-Cyclophophamide-Fluorouracil) and Granulocyte-Colony Stimulating factor breast cancer. Bone Marrow Transplant 22 845-851

26. Chollet P, Charrier S, Brain E, et al. (1997) Clinical and pathological response to primary chemotherapy in operable breast cancer. European Journal of Cancer 33 862-866

27. Koury MJ, Bondurant MC (1990) Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 248 378-381

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