Haematologica 1999; 84:493-498 original paper

Effect of glycosylation of recombinant human granulocytic colony-stimulating factor on expansion cultures of umbilical cord blood CD34+ cells

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ABSTRACT

Backgound and Objective. **The granulocytic colony-stimulating factor (G-CSF) is a cytokine widely used for several purposes such as stem cell mobilization, treatment of neutropenia or *in vitro* cultures. Recombinant human G-CSF (rh-G-CSF) is available in two forms: a non-glycosylated (*E. Coli*-derived), and a glycosylated CHO-derived rhG-CSF. As previously shown, glycosylation gives a higher degree of homology between the recombinant and the wild human G-CSF molecule. This study analyses the role of glycosylation in expansion cultures comparing the biological effects of the two forms of G-CSF.**

Design and Methods. **CD34+ cells from nine cord blood were positive selected (median purity 84%) and cultured in the presence of 50 ng/mL of stem cell factor and 1, 10 or 100 ng/mL of glycosylated rh-G-CSF (GG) or a deglycosylated form (DG). After 5 days of a static, serum-dependent culture fed on day 0, nucleated cells (NC), CD34+ cells and colony-forming units were evaluated and compared using the paired Student's t-test.**

Results. **For all concentrations tested, GG was able to generate more NC and progenitors than DG was able to ($p<0.05$). This effect was mainly observed in CFU-GM colonies, and in CFU-Mix, and indeed no influence was detected in terms of BFU-E expansion. The presence of GG in culture causes the generation of more mature granulocytic cells,assessed by the expression of CD11b/CD15 on CD13+ population, than the presence of DG. In order to check the role of the molecule's stability in this difference, the effect of daily supplementation was tested. Continous presence of cytokines using either form of G-CSF (daily feeding) significantly increased the rate of expansion, but again GG produced higher generation than its DG counterpart.**

Interpretation and Conclusions. **Our results suggest that the stability of the G-CSF molecule has a predominant effect on the higher biological activity found. A glycosylated form of G-CSF is recommended for *in vitro* cultures using serum-dependent conditions.**
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Key words: G-CSF; glycosylation; expansion; hematopoietic progenitor cells; cord blood

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The G-CSF molecule is a cytokine involved in the regulation of, differentiation and maturation of cells of granulocytic lineage.[1] Molecular characterization has allowed it to be purified and obtained through genetic engineering. Recombinant human GCSF was first obtained from bacteria and, in contrast to the wild human molecule, the recombinant polypeptide produced by the bacterial system carries a N-terminal methionine group and is not glycosylated. The suggested role of glycosylation is to increase the molecule's stability, thus protecting it from degradation by unspecific serum proteases, and increasing its half-life. In addition, glycosylation could increase the affinity of the cytokine for the G-CSF receptor, because of the higher degree of homology between the recombinant glycosylated form and the wild G-CSF molecule.[2]

Current manufactured cytokines are produced according to several biochemical specifications, with amino acid substitutions, or with different degrees of glycosylation, or as non-glycosylated forms. These specific differences depend on the type of expression system used to produce the recombinant protein, i.e., bacterial cells (*E.Coli*), mammalian cells (Chinese Hamster Ovary [CHO]) or yeast cells.[3] Recombinant human GCSF (rhG-cSF) is available for clinical uses in two forms: a non-glycosylated form (*E. Coli*-derived) and a glycosylated CHO-derived form, which has 174 amino acids and whose glycosylation part accounts for 4% of the total molecular weight. This glycosylation, a carbohydrate located at position Thr 133, is based in a chain of α N-acetyl-neuraminic acid, β-galactose and N-acetyl-galactosamine.[4]

In vitro studies have highlighted the significance of the sugar moiety of the molecule in conferring greater stability to the rhG-CSF molecule.[5] Deglycosylated rhG-CSF lost most of its activity when pH was increased from 6 to 8, whereas glycosylated rhG-CSF retained almost 80% of its activity.[6] Similarly, the activity of glycosylated rhG-CSF derived from CHO cells was retained after 8 hours' incubation in human serum, whereas that of deglycosylated rhG-CSF was substantially reduced.[7] The molecular mechanism of the increased stability of the glycosylated form remains to be established, but may well

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involve protection of cysteine-17 within the polypeptide chain.[8]

However, the potential clinical advantages of glycosylation still remain unclear. Similarly to interleukin-3, preclinical *in vivo* studies have not shown any important qualitative or quantitative differences in the hematopoietic effects of glycosylated rhG-CSF.[3,9] In contrast, there are *in vivo* biological differences between glycosylated and non-glycosylated forms of other growth factors (GM-CSF and Epo).[10-13]

Several experimental data have shown that glycosylated G-CSF has greater biological *in vitro* activity than deglycosylated G-CSF.[5,14] This difference, as pointed out before, can be explained by a greater stability of the molecule, a higher affinity for the receptor, or both. To refine understanding of the relevance of G-CSF glycosylation we designed a prospective *in vitro* study aimed at comparing the biological activity of a glycosylated and a deglycosylated form of rhG-CSF. This study was based on an expansion culture assay of CD34+ umbilical cord blood (UCB) selected cells. The parameters assessed to determine biological activity were the total number of nucleated cells, CD34+ cells, and colony-forming cells (CFC) generated at the end of culture.

Design and Methods

UCB collection

UCB was obtained from full term pregnancies in the Hospital Universitari Sant Joan de Déu. Signed consent from the mother was required before donation. After early clamping, the umbilical vein was canalized and blood drained by gravity into a standard blood bag containing 25 mL of citrate-phosphate-dextrose-adenine (CPD-A) solution. Bags were stored at 4°C until required for the procedure. All the procedures were started within the 24 hours following collection.

Sample processing and CD34+ positive selection

Within 24 hours of UCB collection, mononuclear cells (MNC) were obtained using a density gradient (Ficoll-Paque, Pharmacia Biotech, Uppsala, Sweden), and were resuspended in a buffered solution (PBS, 1% human serum albumin and 0.5% human immunoglobulin). Positive selection of CD34+ cells, was performed as previously described.[15] Briefly, cells were incubated at a concentration of $1\text{-}5\times10^7$/mL with 0.25 µg of the anti-CD34 MoAb My10 per 10^6 cells, for 30 min at 4°C, with slow agitation. After two washes with buffer 4×10^7 cells/mL were incubated with ferric particles (sheep antimouse-Dynabeads®, Dynal, Oslo, Norway), at a ratio of 0.5 beads per cell. After 30 minutes at 4°C, with slow agitation, the negative fraction was eluted applying a magnetic field (Isolex®-Tm50, Baxter, Deerfiels, IL, USA). Four additional washes were performed to increase the purity of the positive fraction. Cells were detached from the beads (using an enzymatic method) by incubation in the presence of 250 pkat of chemopapain for 15 minutes at room temperature. The reaction was stopped using cold buffer, and afterwards, CD34+ cells were eluted by again applying the magnetic field.

Expansion culture assay

Following positive selection, 10^5 CD34+ cells were seeded in 4-well plates (Nunc, Denmark) in IMDM containing 25% FCS, and incubated at 37°C in 5% CO_2. Cultures were supplemented with several combinations of cytokines. In all cultures 50 ng/mL of stem cell factor (SCF) from R&D Systems (Minneapolis, MN, USA) were added on day 0. Glycosylated rhG-CSF (GG) and a deglycosylated derivative (DG) obtained by enzymatic treatment of the former were kindly provided by Chugai-Rhône-Poulenc (Anthony, France). The amino-acid and amino-sugar composition of these two forms were analyzed to confirm the specifications. Both G-CSF forms were functionally tested in a colony-forming assay and produced similar numbers of colonies. The rhG-CSF concentrations used were 1, 10 and 100 ng/mL depending on the cultures. In the cases indicated, rhG-CSF was added daily. On day +5, assessment of expansion was performed. A series of 9 experiments are presented.

Expansion assessment

Cell expansion was assessed by:

- cell count in a Neubauer's chamber and viability by the trypan blue dye exclusion test;
- *immunofluorescence studies:* expression of CD34, CD11b, CD15 (HPCA-2-PE, D12-PE, MMA-FITC, Becton Dickinson, San José, CA, USA) and CD13 (Immu103.44-PE-Cy5, Coulter-Immunothech, Marseille, France) was determined by flow cytometry on day 0 and day +5. A cytometer (Epics-XL-MCL, Coulter Electronics, Hialeah, FL, USA) was used for acquisition and the results were obtained by analysis of listmodes. For each sample, day 0 cytosetting parameters were defined, and used again for the day +5 analysis in order to compare the intensity of fluorescence for CD34 expression. To assess the mature myeloid cells generated, a gate on CD13+/CD11b/CD15 expression was used: CD11b−/CD15+ defined the *granulocytic population* and CD11b+/CD15+ the *promyelocytic population*;
- semisolid cultures on methylcellulose (1.12%) were performed on day 0 and day +5 using a cytokine cocktail consisting of 10 ng/mL each of IL6, IL3, G-CSF, GM-CSF and 2 U/mL of erythropoietin in IMDM and 20% FCS. Cultures were kept at 37°C, and 5% CO_2 in air, in a humidified atmosphere. CFU-GM, BFU-E and CFU-mix were scored as typical predefined groups of more than 50 cells.[16]

Statistics

Comparison between mean values of different combinations of cytokines for the parameters assessed was analyzed applying the paired Student's

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t-test. The level at which a difference was considered significant was established at $p<0.05$. Results are expressed as mean and standard deviation.

Results

CD34⁺ cell selection

Overall, nine selection procedures were performed, with a median purity of 84%, and a range between 80 to 89%. Cell viability was over 90% in all cases.

Dose-response curve

In order to establish the differences in biological activity between the two forms of rhG-CSF, comparative cultures containing 1, 10 and 100 ng/mL of both cytokines were performed. In these conditions, GG showed a higher biological activity than DG (Figure 1). The differences of the mean in the points analyzed were: 1) NC generation was 3, 2.3 and 2 times greater in GG cultures containing 1, 10 and 100 ng/mL, respectively; 2) similarly, GG allowed $CD34^+$ cell expansion rates 1.9, 1.5 and 1.6 times greater than those produced by DG cultures; and 3) the determination of CFU-GM generated also showed better results when the glycosylated form was used: 1.5, 1.6 and 1.4 times higher expansion rates for the 3 concentrations tested. For all the concentrations analyzed, the differences were statistically significant ($p<0.05$). Wells without cytokines, and with SCF alone failed to expand. In these culture conditions, a combination of cytokines widely used in expansion assays, such as SCF, IL3, IL6 at a concentration of 50 ng/mL each, generated a 7-fold increase in $CD34^+$ cells (data not shown). A trend to a lower difference was observed at 100 ng/mL. These data suggest that the greater biological activity observed using GG could be compensated for a 10-times higher concentration of DG.

The clonogenic activity generated, as assessed in semisolid assays, was also different, showing a greater generation of colonies for a determined concentration in GG supplemented cultures. These differences were mainly observed in CFU-GM counts; no differences were obtained in erythroid colonies. CFU-mix count was highest in cultures supplemented with 100 ng/mL of GG (Figure 2).

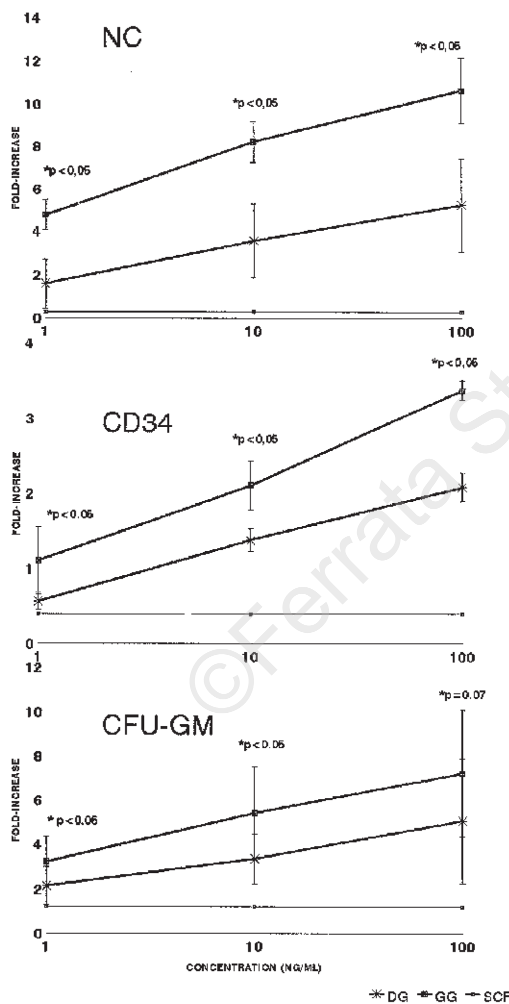

Figure 1. Fold increase (mean and standard deviation of nine experiments) of nucleated cells, CD34⁺ cells and CFU-GM at 1, 10, and 100 ng/mL of glycosylated rhG-CSF (GG) and deglycosylated rhG-CSF(DG). Cytokines were supplemented only on day 0 of culture. The mean of the well supplemented with 50 ng/mL of SCF alone (SCF), used as the control well, is also shown.

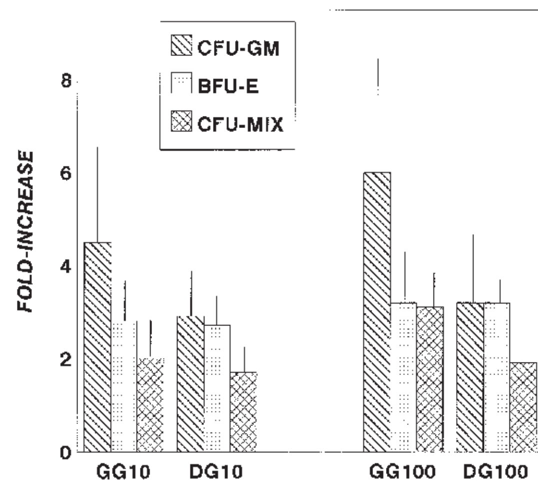

Figure 2. Fold-increase between subtypes of colonies (CFU-GM, BFU-E, and CFU-Mix) according to the concentration used. The type and concentration of G-CSF are abbreviated as follows: GG10 (rh glycosylated G-CSF at 10 ng/mL), DG10 (rh deglycosylated G-CSF at 10 ng/mL), GG100 (rh glycosylated G-CSF at 100 ng/mL), and DG100(rh deglycosylated G-CSF at 100 ng/mL). Cultures were only supplemented at day 0 (see *Design and Methods*). The results are expressed as the mean (and standard deviation) of nine experiments.

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Phenotypic analysis of mature cells generated on day 5 by flow cytometry

On day +5 of the expansion procedure CD11b/CD15 expression was different depending on the G-CSF employed. In GG supplemented cultures, the majority of cells present, and expressing CD13 phenotype, had CD11b+/CD15+ expression while cells from cultures supplemented with the DG form had a higher percentage of cells at a *promyelocytic* stage (CD11b-/ CD15+). The lower biological activity of DG is thus suggested by the delay in terminal granulocytic maturation by this point in the culture (phenotype CD11b+/ CD15+) (Figure 3). This result was also observed using the combination of SCF, IL3, IL6, in a parallel assay. In this case the absence of G-CSF caused a halt in maturation at the *promyelocytic* stage, there being only 8% of phenotypic mature granulocytes after five days of culture. These differences were not observed at 100 ng/mL of G-CSF, suggesting that higher concentrations of deglycosylated G-CSF are needed to achieve the same maturation effects.

Comparative analysis of the effect of daily supplementation of G-CSF in expansion culture

To study the effect of the molecule's stability on these results, we performed five experiments comparing daily cytokine supplementation with standard supplementation (only at day 0) using 1 and 10 ng/mL of G-CSF forms. The effect of daily supplementation in the expansion kinetics and the comparison between the deglycosylated and glycosylated G-CSF are shown in Figure 4. According to these observations, daily supplementation with either form was able to increase biological activity, showing that the continuous presence of cytokines in cultures is relevant for achieving a better expansion efficiency. Nevertheless, in this situation the differences observed in the expansion between 1 and 10 ng/mL using either cytokine are still significantly different. The mean increases of NC generated, directly dependant on the daily supplementation, using DG were 11.9 times at 1 ng/mL and 4.9 times at 10 ng/mL. Using GG the benefits were lower: 4.2 and 2.3 times for 1 and 10 ng/mL, respectively. Instead of a higher increase in biological activity observed with the DG using a daily supplementation schedule, comparison between GG and DG at equal concentrations using a more continuous presence of cytokines still gave better results for the GG form (Figure 4). The differences were still significant when the generation of NC and CFU-GM was analyzed, but the overall expansion of CD34+ cells between these two forms of G-CSF was at the limit of significance ($p=0.05$). According to these results, GG has a greater biological activity than the DG form, probably due to the higher stability of its molecule, since these differences are reduced using a daily feeding protocol.

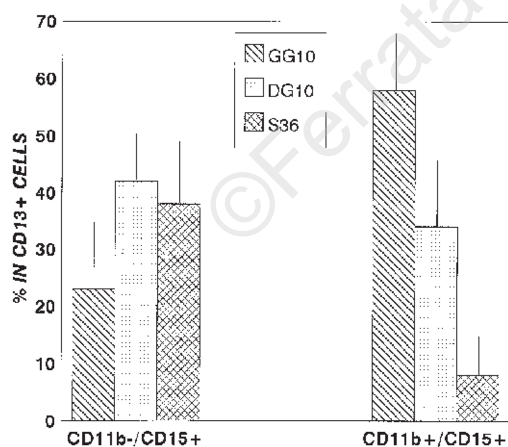

Figure 3. Phenotypic characteristics of mature cells generated in the expansion cultures. The figure compares the percentage of cells expressing CD11b/CD15 on the population expressing CD13 on day +5. DG10 expresses cultures with deglycosylated rhG-CSF at 10 ng/mL, GG10 cultures with glycosylated rhG-CSF at 10 ng/mL and S36 cultures with SCF+IL-3+IL-6 at 50 ng/mL, used as a control of expansion without G-CSF). Cultures were only supplemented on day 0 (see *Design and Methods*). The results are expressed as the mean (and standard deviation) of nine experiments.

Discussion

The availability of cytokine and hemopoietic growth factors has allowed a better understanding of hemopoiesis and its complex regulatory mechanisms. G-CSF is a cytokine extensively used for clinical purposes, specifically for the treatment of neutropenia, and mobilization of hematopoietic progenitors into the peripheral blood.[17-19]

Ideally, polypeptides for clinical use should be identical to those of human origin. The first molecule used as rhG-CSF was produced from bacteria and has several differences with respect to the wild human peptide: the presence of an N-terminal methionine, and the lack of glycosylation. In order to increase the homology between synthetic and wild rhG-CSF, a mammalian cell-derived rhG-CSF (CHO cells) has been produced. Theoretically, GG could improve molecular stability and provide a greater affinity for the receptor.[2] However, strong glycosylation could increase the antigenicity of the molecule, provoking immune reactions against it and causing allergic reactions or drug resistance phenomena,[20] thus failing to reproduce, *in vivo,* the *in vitro* results.

The aim of this work was to study the role of rhG-CSF glycosylation on the *in vitro* biological activity of the peptide. We compared the effect of two forms of rhG-CSF: one, obtained from CHO cells (glycosylated rhG-CSF) and the other, an enzymatic deglycosylated derivative of the first, that has been shown in previous works to have a similar activity to *E. Coli*-derived rhG-CSF.[4] We chose UCB cells to compare

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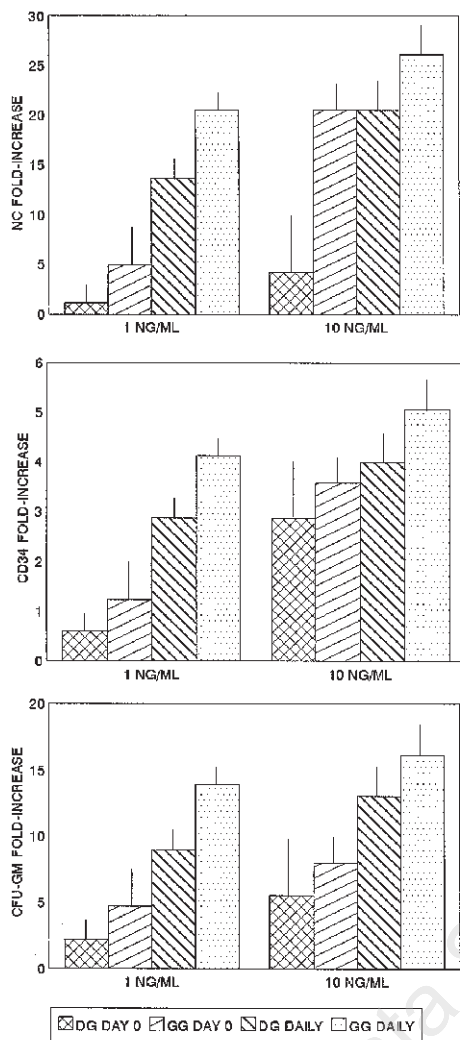

Figure 4. Nucleated cells, CD34+ cells and CFU-GM expansion rate in cultures with glycosylated rh G-CSF (GG) or deglycosylated rhG-CSF (DG) at 1 ng/mL or 10 ng/mL. This figure shows the fold-increase (mean and standard deviation) of each population compared to the starting point according to the feeding protocol employed (supplemented only on day 0 of culture (DAY 0) or with daily supplementation of cytokines (DAILY).

the effects because the previously described[21,22] high proliferative potential of UCB progenitors in response to cytokines, might permit amplification of the hypothetical differences being investigated. Thus, they would allow minimal differences in proliferative activity of hematopoietic progenitors secondary to the action of G-CSF molecule to be observed. The aim of this experiment was not to test G-CSF at the level of stem cell expansion, which requires the use of other early-acting cytokines and conditions. SCF was used to enhance the response and to preserve the viability of CD34+ UCB cells in a five-day assay.[23]

According to the analysis of the dose-response curves at 1, 10 and 100 ng/mL, glycosylated rhG-CSF was superior to the DG form, at each concentration tested. However, adding 10 times more DG than GG abrogated the differences. In contrast to Nissen *et al.*,[5] the differences observed in our experiments were maintained at 100 ng/mL. This could be due to the different assessment of biological activity. Nissen used a clonogenic test to assess biological activity, and thus the effect of the constant presence of cytokine inducing a continuous proliferative stress was not evaluated. Using expansion cultures, a more sensitive approach, we observed that the difference was maintained at high concentrations. Furthermore, different biological activities were observed following the assessment of CFU-GM generated and the phenotypic pattern of granulocytic maturation. To explain our observations we considered two hypotheses: first, the stability of the DG molecule was lower in *in vitro* cultures in which serum was present, and in which the action of non-specific proteases could produce an enhanced elimination curve for the DG molecule. Second, the affinity of the molecule for its receptor could be improved if a greater three-dimensional homology existed between the wild molecule and the rhG-CSF used.[2]

To minimize the role of stability in this phenomenon, we carried out a comparison study with daily supplementation of rhG-CSF. Using this strategy we obtained better expansion rates in all experiments. A continuous presence of active cytokines in the culture medium (achieved by a daily supplementation) produced an overall CD34+ cell expansion 3-times higher than that observed without continuous feeding. These findings suggest that continuous presence of cytokines in the culture plays a positive role in recruiting more progenitor cells into a proliferative state. When daily cultures supplemented with DG and day-0 supplemented GG was compared, similar activity was shown, suggesting stability of the molecules accounted for at least some of the previous difference. In spite of this, cultures supplemented daily with GG still showed greater biological activity than cultures supplemented with the DG molecule counterpart. This difference, however, was lower than that observed with a day-0 supplementation schedule, probably due to a diminished effect of molecule stability. Probably, a more frequent feeding scheme could even eliminate these differences (a feeding interval of 24 hours might be too long, considering the short half-life of the DG molecule in serum-dependent media). A true greater affinity for the receptor could also amplify the expected results observed with GG molecule.

In conclusion, *in vitro* hematopoietic progenitor cell amplification was achieved from UCB CD34+ selected cells. The expansion rates were better using a daily cytokine supplementation schedule. Both G-CSF molecules have a synergistic action with SCF but GG

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yielded better proliferation rates. Our results suggest that the molecule's stability has a major effect in producing the observed higher biological activity. A glycosylated form of G-CSF is recommended for *in vitro* serum-dependent cultures.

Contributions and Acknowledgments

SQ participated in the design and discussion of the study and performed the cultures. JAC participated in the design and discussion of the study and did the flow cytometry. LLA participated in the collection of samples and in discussion of the manuscript. GC participated in the statistical analysis and discussion of the manuscript. JG participated in the design and discussion of the manuscript.

Funding

This work was supported by a grant from Spanish FIS 97/2120 and DGYCYT PB95-0904, and the Fundación Internacional José Carreras para la Lucha contra la Leucemia.

Disclosures

Conflict of interest: none.

Redundant publications: < 50%; resumé of preliminary results in a non-peer reviewed symposium supplement.

Manuscript processing

Manuscript received November 23, 1998; accepted February 19, 1999.

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