RESEARCH

Safety evaluation of granulocyte colony-stimulating factor obtained at CIGB

✉ Lizet Aldana[1], Dania Bacardí[1], Nelson Merino[2], Karelia Cosme[1], Delia Porras[1], Irma Carreras[1], Alfonso Alí[1], José Suárez[1], Ariel Vázquez[1], Yaí Cruz[1]

[1]Center for Genetic Engineering and Biotecnology, CIGB
Ave 31 / 158 and 190, Cubanacán, Playa, PO Box 6162, CP 10600, Havana, Cuba
Fax: (53-7)-271-8070; E-mail:lizet.aldana@cigb.edu.cu
[2]Department of Pathologic Anatomy, Institute of Pharmacy and Food, University of Havana
Street 222 and Ave 23, La Coronela, La Havana, Cuba, PO Box 11600

ABSTRACT

Human G-CSF is a lineage specific colony stimulating factor, which is produced by monocytes, fibroblast and endothelial cells. G-CSF regulates the production of neutrophils from the bone marrow, and it is able to markedly increase the amount of neutrophils in peripheral blood in a 24-hour period. A panel for preclinical studies was designed to evaluate the safety of HEBERVITAL (G-VCSF obtained in Cuba by recombinant DNA technology), before itsuse in clinical studies. It was used in toxicological assays (acute single doses and local tolerance). HEBERVITAL was administered to Sprague-Dawley rats in an acute toxicity study (in which it was compared with NEUPOGEN®, a commercially available product manufactured by Amagen Inc.) as well as in a local tolerance study. Doses that were 150, 300 and 450 times higher than the therapeutic dose were inoculated in the Acute Toxicicty study. The local tolerance study included the therapeutic dose and levels 3 and 10 times higher than that. A control group inoculated with placebo was also included in each study. In fact, no signs of toxicity or histopathologic changes were shown. A local reaction was reported, characterized by subcutaneous granulation tissue as a restoring mechanism. The same finding was reported in animals inoculated with NEUPOGEN. It was concluded that HEBERVITAL is not toxic, and it is well tolerated through the subcutaneous route in the tested dosis in Spaque-dawley rats.

Keywords: lineage specific colony stimulating factor, GCS-F, safety, toxicity

Biotecnología Aplicada 2005;22:50-53

RESUMEN

Evaluación de la seguridad del factor estimulador de colonias de granulocitos producido por el CIGB. El G-CSF humano es un factor estimulador de colonias específico de linaje, producido por monocitos, fibroblastos y células endoteliales. Esta citoquina regula la producción de neutrófilos funcionales de la médula ósea y puede producir incrementos marcados del conteo de neutrófilos en sangre periférica en 24 horas. A partir de la obtención en Cuba de G-CSF (HEBERVITAL) por tecnología de ADN recombinante se diseñó un panel de estudios preclínicos con el objetivo de evaluar su seguridad antes de su empleo en estudios clínicos. El HEBERVITAL fue administrado en ratas Sprague Dawley durante un estudio de toxicidad aguda, en el cual se comparó con un similar comercial de la firma Amgen Inc., California, NEUPOGEN® y en un estudio de tolerancia local. En el estudio agudo se suministró a los animales dosis superiores a la dosis terapéutica, con niveles 150, 300 y 450 veces superiores a ésta. La tolerancia local incluyó, por su parte, la dosis terapéutica y niveles 3 y 10 veces superiores a ésta. Cada estudio incluyó un grupo placebo como control. No se reportaron signos de toxicidad, ni alteraciones morfológicas. Se observó una reacción local caracterizada fundamentalmente por la aparición de tejido de granulación subcutáneo como mecanismo de reparación; resultados similares a los reportados para los animales inoculados con NEUPOGEN®. Concluimos que en el espectro de dosis explorado en ratas Spague-Dawley, el HEBERVITAL administrado por vía subcutánea es clasificado como no tóxico y tolerable.

Palabras claves: factor estimulador de colonias específico de linaje, GCS-F, seguridad, toxicidad

*I*ntroduction

Granulocyte-Colony Stimulating Factor (G-CSF) is a 174 -amino acid protein with a molecular weight of 18-22 kDa [1] whose gene is located in the q21-22 region of chromosome 17. Its structure is composed of anti-paralell helices and has two disulphur links between C_{39}-C_{45} and C_{67}-C_{77} cysteins and glycolization in treonin 133 which contributes to its stability. Colony stimulating factors act by linking to their 140 kDa [1-4]) cell receptors. The introduction of recombinant DNA technology has allowed the large scale production of this factor. G-CSF is obtained by the insertion of the human gene which codifies it in *Escherichia coli (E coli)*. It is then purified following good manufacturing practices for biopharmaceuticals [5]. The G-CSF brand now produced in this way at the Center for Genetic Engineer and Biotechnology (Havana, Cuba) is known as HEBERVITAL. It has been most extensively used in bone marrow implants and its application in neutrophilic either chronic or induced by chemiotherapy has been widely studied. It is known that G-CSF also accelerates neutrophilic recovery after bone marrow implants and in patients with acute leukemia. The posibility of its use in patients with metastatic breast cancer, multiple myelomas, Felty's syndrome, bacterial infections, and in AIDS patients, is reported as well [6-8]. On the other hand, the possibility of using G-CSF in combined therapies with other cytokines for diverse pathologies, is currently being

1. Burke F, Naylor MS, Davies B, Balkwill F. The cytokine wall chart. Immunology Today 1993;14(5):165.

2. Hamilton JA. Colony stimulating factors, cytokines and monocyte-macro-phages-some controversies. Immunology Today 1993;14(1):18-24.

3. Fresno M, Kopf M, Rivas L. Cytokines and infectious diseases. Immunology Today 1997;19(3):57.

4. Pistoia V. Production of cytokines by human B cells in health and disease. Immunology Today 1997;18(7):343-50.

✉ Corresponding author

assessed. The most common treatment dose is 5µg/kg body weight through the subcutaneous route [6].

G-CSF has been used in numerous preclinical and clinical studies. NEUPOGEN®, (G-CSF manufactured by Amgen Inc, California) was shown to be safe for its use in humans, after being administered to non human primates, dogs, hamsters, rats, and mice in acute, subacute, and chronic toxicology studies [9-11]. Clinical studies have shown a dose -dependant increase of circulating neutrophil counts by using 1-70 mcg/kg/day dose ranges as well as some adverse events that have not invalidated its therapeutic use [7, 8, 12].

Clinical studies are now planned in Cuba, due to the importance of placing HEBERVITAL on the market. however, pre-clinical studies are first needed to evaluate this product´s safety. Hence, results on acute toxicity and local tolerance studies are shown in this paper.

Materials and methods

The studies were carried out according to the ethical standards established for the use of laboratory animals [13], following Good Laboratory Practice guidelines[14, 15] and Operation Pattern Procedures approved for toxicology studies.

Animals used in the study

Sprague-Dawley rats (Subline Cenp: SPRD *ALY*®), were supplied by the gnobiotic rodents division of the National Center for the Production of Laboratory Animals (known as CENPALAB). The animals were subjected to a clinical check up on arrival; they were weighed and housed in individual makrolon boxes (Tecniplast Italy) with sterile sugar cane bagasse pith beds. They were observed for 7 days before starting the study, at controlled environmental conditions (19-21 °C, 68% average relative humidity and 12 -hour light- darkness cycles) which remained constant during the experimental phase. The animals were given, 25 g per animal of *ALY co*, *(CENPALAB*) daily and water ad libitum. Fifty rats, of 5-6 weeks of age (25 of each sex) were selected for the acute toxicity study. They started the study with an average body weight of 134,75g ± 0.55. The local tolerance study used 40 young adult males of 158. 08 g ± 2. 6.

Formulation

HEBERVITAL lots, manufacured by Center for Genetic Engineering and Biotechnology (Havana, Cuba) were used in both studies. They were 99% pure (HPLC-RP), a biological activity of 70. 4 x 10^6 IU/mL, and a protein concentration of 0.39 mg/mL (Lowry). They had good sterility, pH, volume and product stability. NEUPOGEN manufactured by Amgen Inc. was obtained from a commercial lot.

Evaluation of Acute Toxicity. A comparative study with NEUPOGEN

Experimental design

This study was designed according to regulations established by ICH/EMEA International Conference on Harmonization/European Agency for the Evaluation of Medicinal Products[16, 17]. Five working groups were formed with 10 animals each (5 of each sex). The following treatments were administered parenterally by the subcutaneous route (Table 1).

Once the drug was administered, all rats were observed daily to record changes in behavior or signs of toxicity. These clinical evaluations were performed according to the evaluation method described by DiPascuale and Hayes [18] and included changes in skin and hair, coloring and appearance of the mucose membranes and eyes, in the respiratory, central nervous, and autonomous systems as well as in somatomotor activity. Body weight was measured at days 1, 7, and 14 of the study and food consumption was quantified daily. Animals were slaughtered by cervical dislocation after anesthetic treatment with ether. They were bled by cutting the femoral artery. A macroscopic observation of all organs was made during the necropsy and samples of the liver, spleen, mesenteric ganglia, and administration site were collected for histopathological evaluation. These samples were processed using hematoxiline-eosine and observed with an Olympus microscope (Japan).

Evaluation of local tolerance

Experimental design

The study was designed according to aspects described in the ICH/EMEA guideline 2145/00 [19]. Four treatment groups were formed with 10 animals each as shown in table 2.

The dose was administered subcutaneously, every day, for 7 consecutive days except for 5 animals of each group that were slaughtered at day 2, after just one HEBERVITAL subcutaneous inoculation. During the daily clinical observations special emphasis was placed on finding the local damage produced by the tested substance. The procedure described above was followed to slaughter animals and macroscopic observations were made and samples collected from the liver, spleen, mesenteric ganglia, and the administration site for a histopathologic study. Body weight was individually recorded at days 0 and 8 and feed consumption was measured daily.

Data processing

Variables used for statistical analyses were body weight (BW), average weekly feed consumption (FC),

5. Tatsuda D, Aimura H, Tokunaga H, Ishibashi M, Arakawa T, Tokunaga M. Expression and purification of citokine receptor homology domain of human G-CSF receptor fusion protein in *E Coli*. Protein Expr Purif 2001;21(1):87-91.

6. Neidhart J, Mangalik A, Kohler W, *et al*. Granulocyte colony-stimulating factor stimulates recovery of granulocytes in patients receiving dose-intensive che-motherapy without bone-marrow trans-plantation. J Clin Oncol 1989;7:1685-91.

7. Gabrilove JL, Jakubowski A, Fain K, *et al*. Phase I study of granulocyte colony-stimulating factor in patients with transitional cell carcinoma of the urothelium. J Clin Invest 1988;82:1454-61.

8. Morstyn G, Souza L, Keech J, *et al*. Effect of granulocyte colony-stimulating factor on neutropenia induced by cytotoxic chemotherapy. Lancet 1988;1:667-72.

9. Neupogen®. Product Information. Amgen Inc., California. On line: www/neupogen.com.2002.

10. Zsebo KM, Cohen AM, Murdock DC, *et al*. Recombinant human granulocyte colony-stimulating factor: Molecular and biological characterization. Immunobiol 1986;172:175-84.

11. Welte K, Bonilla MA, Gillio AP, *et al*. Recombinant human G-CSF: Effects on hematopoiesis in normal and cyclophosphamide treated primates. J Exp Med 1987;165:941-8.

12. Bronchud MH, Scarffe JH, Thatcher N, *et al*. Phase I/II study of recombinant human granulocyte colony-stimulating factor in patients receiving intensive chemotherapy for small cell lung cancer. Br J Cancer 1987;56:809-13.

13. Guidelines for breeding and care of laboratory animals World Health Organization and Internacional Council for Laboratory Animals Science (ICLAS). 1998.

14. Food and drug administration. Good Laboratory Practice for non clinical laboratory studies. Title 21 Code of Federal Regulations, Subchapter A, Part 58, 1997.

15. Programa para el uso de animales de experimentación del Centro de Ingeniería Genética y Biotecnología. U Bioterio, CIGB, 1998.

Table I. Treatment groups in the acute toxicity study

Group	Treatment	Dose (mg/kg)
I	Placebo	-
II	150 times the HEBERVITAL therapeutic dose	0.75
III	300 times the HEBERVITAL therapeutic dose	1.50
IV	450 times the HEBERVITAL therapeutic dose	2.25
V	450 times the NEUPOGEN therapeutic dose	2.25

Tabla 2. Treatment group in the local tolerance study

Group	Treatment	Dose (µg / kg)
I	Placebo	-
II	HEBERVITAL therapeutic dose	5
III	10 times HEBERVITAL therapeutic dose	50
IV	30 times HEBERVITAL therapeutic dose	150

and histopathologic findings (HF). In all cases central trend and dispersion measurements (mean, standard deviation, maximum and minimum values) were estimated. Normality assumptions (Kolmogorov-Smirnov's and Shapiro-Wilk's tests) and variance homogeneity (Levene' s test) were verified for BW and FC analyses, at each evaluation, and for each sex by applying a parametric variance analysis (ANOVA) or the non-parametric alternative (Kruskall-Wallis's test). Paired comparisons were made in consecutive intervals by using t paired tests or Wilcoxon's test, for the fulfillment of the assumption of a normal distribution. Data resulting from the histopathology study were analyzed by crossed classification tables with the associated independence test (Fisher's accurate test) [20] . Data processing was carried out using the SSPS8. 0 program on Windows [21]

Results

Clinical observations

No clinical signs of acute toxicity or adverse effects caused by the substance, were reported as a result of daily observations in both studies. No changes were detected in hair color and appearance of the eyes and mucose was normal as well as the somatomotor activity and behavior with an adequate response to stimuli and no deaths were reported.

No erythema and / or edemas were observed, or were there any other signs of local irritation or any other damages attributable to the administration of HEBERVITAL at the administration site during the local tolerance study.

Body weight

The acute toxicity study showed an increase in body weight with time when results were compared for days 0 and 14; females however showed a decrease in this parameter during week 2, differing from males that gained weight constantly (table 3). This parameter was also analyzed according to the dose administered at each evaluation time and no significant differences were shown between groups (day 7, p 0. 089; day 14, p 0. 075).

This parameter showed a significant increase in the case of local tolerance, with no significant differences reported between groups for an equal weighing period. This also indicated that dose variation did not affect live weight gains (table 4).

Feed consumption

Feed consumption remained withing the ranges established for healthy rats (17-25 g/day) [22]. The comparative study between HEBERVITAL and NEUPOGEN did not show differences in this parameter between animals treated with both products, although a variation in feed consumption is evident in both males and females, with an increase at week 2. Later consumption remained stable, within the above mentioned ranges.

Macroscopic and histopathological evaluation

Macroscopic obsevations performed during necropsy in both studies did not show alterations in organs and tissues, but the presence of local hemorrhagic sites found in the local tolerance study are the result of the trauma caused by inoculation.

The histopathological evaluation showed very similar results in both studies. A slight reactive hepatitis with Kupfer's cell hyperplasia and focal aggregates of mononuclear inflammatory cells (figure 1) was detected. This condition was shown by 2 animals of groups I and II, 3 of Group III, 5 of Group IV, and 6 of GroupV. All treatment groups also showed lymphoid hyper reactivity in spleen and lymphatic glands with active germinal centers.

The histopathologic findings at the administration site, during the local tolerance study, may be analyzed at two moments: 24 hours after the first administration and at day 8, after 7 successive HEBERVITAL subcutaneous inoculations. Findings reported at 24 hours are shown in table 5 where it may be observed that just a few animals show local alterations. Figure 2 shows the main effects found.

No local adverse events attributable to the substance under study or to the placebo, were reported as a result of the histologic evaluation performed after 7 administrations of HEBERVITAL. No group showed

Table 3. Body weightl (g) male and female animals. acute toxicity study

Groups	Day 1		Day 7		Day 14	
	Female	Male	Female	Male	Female	Male
I	131.4±5.86	139.2±7.60	195.4±5.59	164.8±5.45	172.0±12.1	210.8±11.5
II	130.6±6.66	138.0±8.00	194.8±6.06	165.2±6.42	167.4±6.43	210.4±6.43
III	130.0±4.18	139.2±6.69	196.0±5.92	167.6±5.77	173.4±7.09	214.2±9.34
IV	131.4±7.70	138.4±9.45	200.6±4.04	168.8±4.97	172.4±10.7	212.2±5.26
V	130.8±6.30	139.4±10.5	198.4±3.91	162.6±6.99	170.2±9.73	203.4±4.72

Table 4. Body weight (g) of animals included in the local tolerance study

Group	Day 1	Day 7	p
I	156.80±13.08	170.60±22.30	0.043
II	157.00±10.65	167.40±7.27	0.015
III	156.60±12.70	167.60±15.81	0.004
IV	162.00±11.07	174.20±12.46	0.039

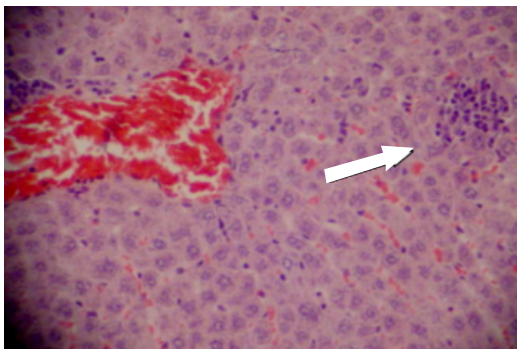

Figure 1. Reactive Hepatitis reported by acute toxicity study. Local lymphoid aggregates are shown (arrow) 100X.

16. ICH/EMEA. Preclinical safety evaluation of Biotechnology-Derived Pharmaceuticals. Step 4. (CPMP/ICH/302/95), 1997.

17. ICH/EMEA. No-clinical safety studies for the conduct of Human clinical trials for pharmaceuticals. ICH M3 (M) (modification of CPMP/ICH/286/95), 2000.

18. DiPasquale L, Hayes W. Acute Toxicity and Eye Irritancy. In: Principles and methods of toxicology. Taylor and Francis (USA) 2001. (Hayes W ed., Fourth Edition). p. 864-7.

19. ICH/EMEA (2001). Note for Guidance on non-clinical local tolerance testing of medicinal products CPMP/SWP/2145/00. Animal use and toxicity evaluation. Victor S and Lucas JR Ed, New York, 1994. p. 29.

20. Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance Biometrika 1988;75:800-2.

21. SPSS Inc. Statistical Package Scientific System, version 8.0, Windows. 1997.

hepatic damage. Lymphoid hyperplasia was detected in all groups which is more evident in lymphatic ganglia with paracortex expansion and the formation of active germinating centers (Figure 3). No signs of damages caused by G-CSF were found in the evaluation of the irritating capacity on muscular tissue.

Discussion

Neither the acute toxicity nor the local tolerance studies showed toxicity signs or alterations in behaviour. These studies showed variations in feed consumption and body weight, mainly in females, but this did not mean that there were substantial bilogical alterations because, in general, they gained weight and consumed feed within the parameters reported for healthy rats (17-25 g) [22]. Clinical observations showed hemorrhagic areas in animals included in the local tolerance study (hemorragic) which were not due to the substance under study but to the method and the repeated administration, since it was also observed in animals inoculated with placebo.

Signs of immunologic and cellular stress detected at the administration site corresponded to animals with repeated administration of HEBERVITAL. The pattern of inflammatory reactions, seems to be caused by the administration of high volumes and doses of the tested product which agrees with other authors who have mentioned hemorrhagic foci with lymphoid infiltrations after the administration of high doses of G-CSF [9, 10].

It is important to point out that the findings reported in the acute toxicity study were the same for groups inoculated with HEBERVITAL and NEUPOGEN®. Hepatic response seems to be related to the G-CSF self pharmacologic action mecanism because the presence of mononuclear focal aggregates and Kuppfer´s cell hyperplasia increases in frequency with the dose. The liver histopathologic test in studies with several species showed an increase of extramedular granulopoiesis and hyperplasia as a res-ponse to the G-CSF pharmacologic action [9]. Augmented splenic response and lymphoid hyperreactivity detected in the lymphatic nodes as well as in the spleen of animals included in the local tolerance study that agrees with reports from other studies [10, 11] in which hyperplasia in the liver and spleen was shown as a result of a dose-dependant extramedular granulopoyesis induced by G-CSF.

In general these local and systemic findings are not considered toxic signs and seem to be a consequence of the high dose levels administered as referred to in the literature [3, 4, 6]. The fact that most of the animals did not show alterations favors the safety the trial subs tance and suggests that the dose must be taken into account to ensure it. HEBERVITAL had a response pattern similar to that obtained with NEUPOGEN®, a product that has been registered in many countries with a high commercial and therapeutic impact. Our results lead to the conclusion that HEBERVITAL, in the dose spectrum explored, produces effects given by its action mechanism in organs such as the liver, lymphatic nodes, spleen, and at the administration site. These effects are not considered to be signs of toxicity.

Although HEBERVITAL and NEUPOGEN® differ in their formulations, results obtained during the experimental phase and the histopathologic study were similar.

Table 5. Microscopic finding reported in animals performed 24 hours after administration

Groups	Without alterations	Subcutaneous granulation tissue	Local inflammatory reaction	Necrosis
I	1/5	0/5	3/5	1
II	4/5	0/5	1	0
III	2/5	1/5	3*	0
IV	2/5	2/5	2*	0

*Local inflammatory reaction at the administration site in the initial damage restoring phase

22. Victor S. Animal use and toxicity evaluation. Lucas JR (ed) 1994. p. 29.

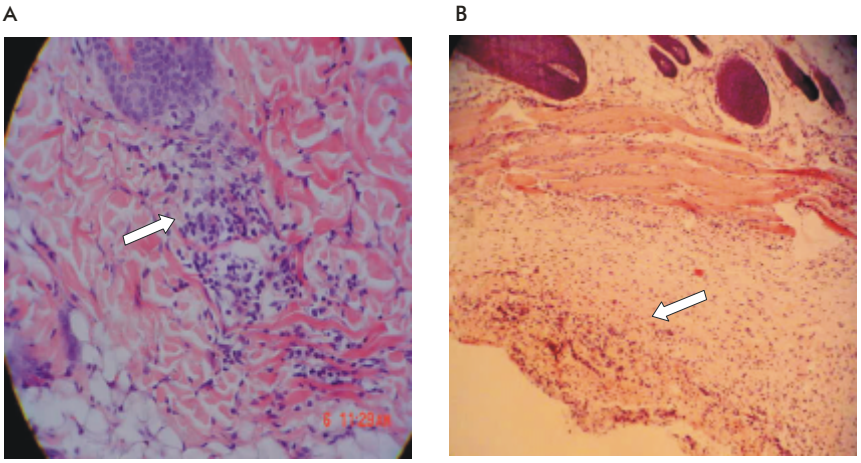

Figure 2. Microscopic finding reported during Local tolerance study. A:Dermal Inflammatory reaction (arrow); B:Subcutaneous granulation tissue (arrow) 100X.

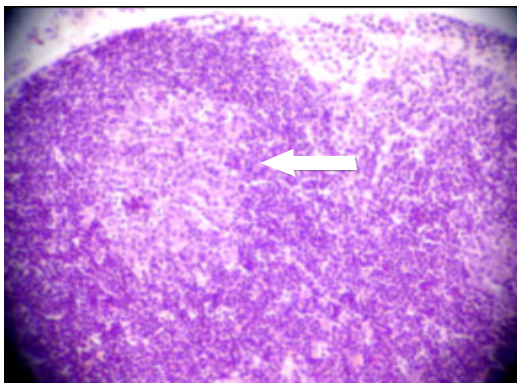

Figure 3 . Local tolerance study. Lymphoid hyperplasia in mesenteric lymphatic node . The germinative center is shown (arrow) 100X.

Received in december, 2003. Accepted for publication in october, 2004.