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In vitro bioassay with enhanced sensitivity for human granulocyte colony-stimulating factor

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Abstract: A method for the determination of human granulocyte colony-stimulating factor (hG-CSF) activity, based on stimulation of cellular proliferation, was developed using a subclone of the murine myeloid leukemia cell line NFS-60, with an improved sensitivity for hG-CSF, as indicator. The optimal range for quantitative analysis of hG-CSF was about 4–60 pg ml^{-1}. The stimulatory effect was measured by a colorimetric microassay: the optical density of formazan, which is produced by viable cells from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was obtained by reading plates in a multi-channel photometer. The assay was designed as a five-dose parallel line test, employing three or four doses for potency determinations, which fulfil pharmacopoeial requirements for assay validity. Inter-assay relative standard deviation (RSD) varied between 5.2 and 12.0%. Most assay experiments revealed potencies within limits of error of 90–110% and the mean index of precision value was 0.057. The recently developed yeast cell-derived International Standard (88/502) served as a reference for activity of rhG-CSF. Specificity of the assay was demonstrated by absence of response upon exposure to a panel of biomolecules, including recombinant human interleukin-3, and by the suppression of growth stimulation in the presence of neutralizing anti hG-CSF antibodies. Potency readings of unglycosylated rhG-CSF were dependent on pH of assay medium with higher relative activities observed at pH 6.6 than at 7.4. Moreover, SDS-PAGE analysis of the carbohydrate-deficient preparation, following incubation at physiological pH, revealed several high molecular weight rhG-CSF bands and decreased monomeric form. The method described was found suitable for potency assessments of pharmaceutical formulations of hG-CSF.

Keywords: *G-CSF; bioassay; formazan; NFS-60; specificity.*

Introduction

The proliferation, differentiation and viability of hematopoietic cells is under the influence of the colony-stimulating-factor (CSF) family of polypeptides [1]. The development of semi-solid culture systems [2, 3] has hitherto enabled the identification of four CSFs: macrophage-, granulocyte-, granulocyte-macrophage- and multilineage-CSF, also denoted interleukin-3 (M-, G-, GM- and IL-3, respectively), each with a distinct type of stimulatory action on progenitor cells in the bone marrow [4]. The CSFs also modulate functional activities of mature target cells. Whereas IL-3 and GM-CSF have a broad spectrum of responsive hematopoietic cells, the G- and M-CSF factors are considered as comparatively lineage-specific stimulators [1].

First identified in lungs from endotoxin-treated mice, G-CSF was shown to promote differentiation of the murine myelomonocytic leukemia cell line WEHI-3B(D+) [5, 6]. Following purification of the human counterpart from cell-conditioned media, it became evident that G-CSF from both species displayed comparable biological and receptor-binding properties [7–11]. G-CSF is distinguished by its ability to preferably stimulate the formation of granulocytic colonies in cultures of hematopoietic stem cells, and to enhance neutrophilic chemotactic and microbicidal abilities [8, 12]. Human G-CSF is produced in very limited amounts *in vivo*, as are all members of the CSF family [1]. However, the molecular cloning and cDNA expression of hG-CSF [10, 13, 14] has greatly facilitated research on its effects on the hematopoietic system, which has resulted in a deeper understanding of the biology of this cytokine. (For a review, see [15].) The stimulatory actions on myeloid progenitor cell proliferation and maturation have been utilized in the treatment of various conditions of myeloid cell deficiency and/or malfunction. These include congenital and idiopathic neutropenic disorders as well as bone marrow transplantation [16, 17]. Moreover, patients under

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chemotherapy regimens have experienced a faster neutrophil recovery with rhG-CSF [18, 19]. Thus, rhG-CSF, expressed in *E.coli* or in mammalian cells, has been approved for marketing in several countries which has contributed to a need for methods that allow reliable determinations of G-CSF bioactivity.

We have developed and evaluated an *in vitro* bioassay for rhG-CSF, based on its stimulatory effect on proliferation, using a clone of the murine myelomonocytic cell line NFS-60 [20]. Results are obtained by optical readings of the coloured formazan product, which is a cleavage product of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in viable cells [21], using a microtiter-adapted photometer. Thus, extensive sample processing steps and radionuclide utilization are not needed. The potencies of G-CSF preparations are determined as a percentage of the activity of the recently established yeast cell-derived International Standard for Human Recombinant G-CSF [22] (generic name: sargrastim). The assay has been designed to fulfil pharmacopoeial requirements for the quantification of pharmaceutical preparations of biologicals, but would be applicable for the analysis of hG-CSF from other sources.

Materials and Methods

Reagents

The International Standard for Human Recombinant G-CSF (88/502; yeast cell derived; generic name: sargrastim) was provided by the National Institute for Biological Standards and Control (Potters Bar, Hertfordshire, UK). This material was stored in sealed ampoules at −20°C and transferred to −70°C in small aliquots, following reconstitution in assay medium. rhG-CSF, expressed in Chinese Hamster Ovary cells (lenograstim) and *E.coli*-derived rhG-CSF (filgrastim) were provided by two manufacturers. A 6.0 mg ml^{-1} stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; the Sigma Chemical Co., St Louis, MO, USA) was sterilized by filtration and stored in a light-protected container at +4°C. It was discarded after one month. HEPES buffer was from Flow Laboratories, Irvine, UK. rhIL-2 and rhIL-3 were gifts from EuroCetus B.V. (Amsterdam, the Netherlands) and from Sandoz Pharma Ltd (Basel, Switzerland), respectively. Promega Corporation (Madison, WI, USA) and Boehringer–Mannheim GmbH (Mannheim, Germany) were suppliers of rhIl-4 and rhIL-6, respectively. Human insulin (Actrapid®), human growth hormone (GH, Norditrophin®) were both from Novo Nordisk A/S (Bagsvaerd, Denmark). rhErythropoietin (Epo, Recormon®) was obtained from Boehringer–Mannheim GmbH, and rhGM-CSF (Leukomax®) from Sandoz Pharma Ltd. Murine monoclonal antibodies towards human G-CSF (Ab-2) were purchased from Oncogene Sciences Inc. (Manhasset, NY, USA). Peroxidase-conjugated rabbit anti-mouse immunoglobulins and monoclonal murine antibodies against human IL-3 were obtained from Genzyme Corp. (Cambridge, MA, USA).

Cell culture

NFS-60, a myeloid murine leukemia cell line, was kindly provided by Dr J. Ihle, St Jude Children's Research Hospital, Memphis, TN, USA. The cells were maintained in RPMI 1640 medium (The National Veterinary Institute, Uppsala, Sweden), supplemented with 10% foetal calf serum, 4 mM L-glutamine, 100 IU ml^{-1} benzylpenicillin, 100 μg ml^{-1} streptomycin (Biochrom KG, Berlin, Germany) and 10 μM β-mercaptoethanol. rhG-CSF, 15 IU ml^{-1}, was also included in the medium. Cells were maintained in a humid atmosphere, with a composition of air–CO_2 (95:5), in 50-ml polystyrene Nunclon Delta flasks (A/S Nunc, Roskilde, Denmark) and split three times per week.

Cell density determination

From each microwell culture (each microculture was adjusted to 210 μl shortly before this step) 200 μl was diluted 100-fold with isoton II solution (Coulter Electronics Ltd, Luton, UK). Samples were analysed for cell numbers (three readings each) using an automatic cell counter (Coulter Counter, D Industrial from Coulter Electronics Ltd). Each dose tested was analysed in pentaplicate.

Colorimetric assay

Before each experiment, the cells were washed three times in ice-cold phosphate-buffered saline (PBS) and resuspended in culture medium with rhG-CSF excluded (assay medium, standard type). Solutions of the test substances were subjected to serial dilutions in

standard or modified (see below) assay medium, transferred in pentaplicates (90 μl each) to flat-bottomed microtiter plates (Nunclon Delta SI, A/S Nunc, Roskilde, Denmark) and mixed with an equal volume of the prewashed cell suspension to a final density of 5.0×10^4 cells per ml. Plates were then incubated at 37°C for 48 h before addition of 20 μl of the MTT solution. The reaction was allowed to proceed during 4 h in an incubator and was quenched by 70 μl of an acidified solution (pH 2.65) of 25% sodium dodecyl sulphate (SDS). In some potency determination experiments, the assay medium was adjusted by the addition of HEPES (Flow Laboratories, Irvine, UK) to 25 mM final concentration and a subsequent adjustment to pH 6.6, using HCl as a titrator. Such reactions were terminated with unacidified SDS solution. The plates were left overnight at room temperature (protected from light), which allowed dissolution of formazan crystals, whereupon the absorbance values were determined on a multi-channel photometer (SLT 340 ATTC, SLT-Labinstruments, Salzburg, Austria). Readings were conducted at 570 and 620 nm as test and reference wavelengths, respectively [21, 23, 24]. The arithmetic mean values of three readings (OD_{RES}) were used for processing, potency determinations and graphic representations, according to the following formula

$$OD_{RES} = (OD_{570}-OD_{620}) - (OD_{570BLANK}-OD_{620BLANK}).$$

Dose–response analysis. Two-fold dilutions of rhG-CSF were prepared, transferred to microtiter plates and incubated with target cells, as described above. When antibodies against hG-CSF, or with specificity to other cytokine, was included in the experiment the diluted samples were subjected to 30 min preincubation before contact with indicator cells.

Potency determination. Preparations of rhG-CSF were subjected to 1.6-fold dilutions and loaded on microtiter plates in pentaplicates. The International Standard for Human Recombinant G-CSF was employed as reference for potency. Concentrations used were selected from the linear range of the log dose–response curve.

Western analysis

rhG-CSF from various sources were subjected to separation by SDS-PAGE (12.5–15.0% gradient gel), using the Mini-Protean II system (Biorad Laboratories, Richmond, CA, USA), essentially as outlined [25]. Each sample contained an estimated amount of 8 ng (0.4 pmol) cytokine. Proteins were transferred to 0.45 μm nitrocellulose membranes (BA 85, Schleicher & Schuell, Dassel, Germany). Blocked membranes were exposed to mouse monoclonal anti-hG-CSF antibodies (diluted to 1/200 of the stock concentration in PBS with 0.1% Tween-20 and 0.5% bovine serum albumin included) and subsequently to peroxidase-conjugated anti-mouse immunoglobulin G antibodies (diluted 2000 times in the aforementioned buffer), with several washes in between. Membranes were subsequently treated with luminol and peracid reagents (Amersham, UK); induced luminescence was captured on photographic film (Kodak XAR-5, Eastman Kodak Co., Rochester, NY, USA) and developed using standard procedures. Prestained marker proteins (Low-range, Biorad Laboratories, Richmond, CA, USA) were employed as standards for molecular weight.

Densitometry

Exposed X-ray films were read on an XRS-6cx flat bed scanner (X-Ray Scanner Corporation, Torrance, CA, USA). Obtained images were processed by the Bioimage software module for 1-D gel analysis (Millipore Corporation Imaging Systems, Ann Arbor, MI, USA), using a SPARC IPC workstation (SUN Microsystems Inc., Mountain View, CA, USA).

Data processing

The potency of each test preparation was calculated by analysis of variance for three or four-dose assays according to principles described in the European Pharmacopoeia [26]. The statistical weight is defined as the reciprocal value of the variance of the $\log_{10}$ potency estimate [26]. The index of precision was obtained by dividing the standard deviation of the responses by the negative slope of the $\log_{10}$ dose–response relationship [27]. After testing for homogeneity, the weighted $\log_{10}$ potency estimates were employed for combination of results from separate assays [26]. The RSD (coefficient of variation) is defined as

the ratio between the standard deviation of the responses and the arithmetic mean value of these responses.

Results

Cellular response studies

Human G-CSF has been reported to induce a proliferative response in the murine myeloid leukemia cell line NFS-60 [20], through binding to a high-affinity specific receptor [28, 29]. It was found that the sensitivity to recombinant human G-CSF (rhG-CSF) increased when the cells were constantly exposed to a low concentration of this cytokine. Following maintenance for 6 months in 8 pM concentration of rhG-CSF, the threshold level of detection changed from about 1.5–3.0 IU ml^{-1} (0.8–1.6 pM) to 0.2–0.4 IU ml^{-1} (0.1–0.2 pM) (Fig. 1A). A linear log dose-response range encompassed from 0.5 to 6.12 IU ml^{-1} of rhG-CSF (Figs 1 and 2). Stimulation to growth was confirmed by electronic cell counting. Dose response curves obtained with this method were similar to those of the colorimetric assay (Fig. 1B). The described indicator line is henceforth referred to as NFS-60S. As the parental NFS-60 line has been characterized as susceptible to murine IL-3 [30], the cellular proliferation upon contact with the human counterpart was measured. However, no reaction was obtained upon exposure to rhIL-3 when using our standard 48 h stimulation-assay. Recombinant hIL-2 and rhIL-4 were also devoid of growth-promoting impact, whereas, rhIL-6 induced a faint response at high concentration levels. When assayed over a broad dose-range, none of the following substances (all of recombinant human type) were able to support growth of NFS-60S cells: insulin, somatotropin, erythropoietin and granulocyte-macrophage colony-stimulating factor (not shown).

The specificity of the assay was further demonstrated by pretreatment of rhG-CSF with monoclonal anti-hG-CSF antibodies: an increasingly more pronounced suppression of the response paralleled the gradually elevated antibody load and resulted in an extinction of stimulation at the highest titer of the neutralizing reagent (Fig. 2). When monoclonal antibodies against hIL-3 was employed in a similar experiment, no inhibition of the rhG-CSF induced response was observed (not shown).

Previous reports have documented a considerable loss of biological activity upon dilution of polypeptide factors to low concentrations in protein-poor solutions as a result of adsorbance to plastic surfaces [31, 32]. To determine a putative requirement of additional desorbants in our assay system, the standard preparation of rhG-CSF was diluted with different concentrations of bovine serum albumin (BSA) in separate experiments. However, elevated protein contents in the dilution/assay medium did not produce appreciable alterations of the stimulatory potency of rhG-CSF at concentrations within the employed range. Conclusively, the used content of foetal calf serum (10%) in response studies and potency determinations, provided sufficient protein amounts for efficient occupancy of unspecific plastic binding sites.

Potency determinations

In order to evaluate the precision and inter-assay variation of the bioassay the potencies of an rhG-CSF preparation, diluted to various known concentrations, was determined. Thus, two separate sample series were treated in parallel and five doses, from the linear response range, were selected for the MTT colorimetric assay. In each set of experiments, pentaplicate samples were used. Typical effects are depicted in Fig. 3 and the results of statistical analysis of responses to three or four doses, are summarized in Tables 1 and 2. Discrepancies between nominal and estimated potencies of rhG-CSF were generally small and the fiducial limits were narrow. Moreover, the index of precision was below 0.100 in all of these estimations. Since the low intra-assay variation, small deviations from linearity or parallelism sometimes became statistically significant (see Fig. 3). When such results were obtained, the four-dose analyses were replaced by three-dose equivalents. Accordingly, a maximal part of the linear response was consistently used for data processing. The main inter-assay RSD, as obtained from three experimental subgroups, was 7.5%. Whenever possible the weighted log potency estimates, from experiments within each such assembly, were combined (Table 2) [26].

Two commercial formulations of rhG-CSF, including three separate batches of preparation 1, were also determined using the International Standard (WHO 88/502) as a reference for activity. The assays were conducted as

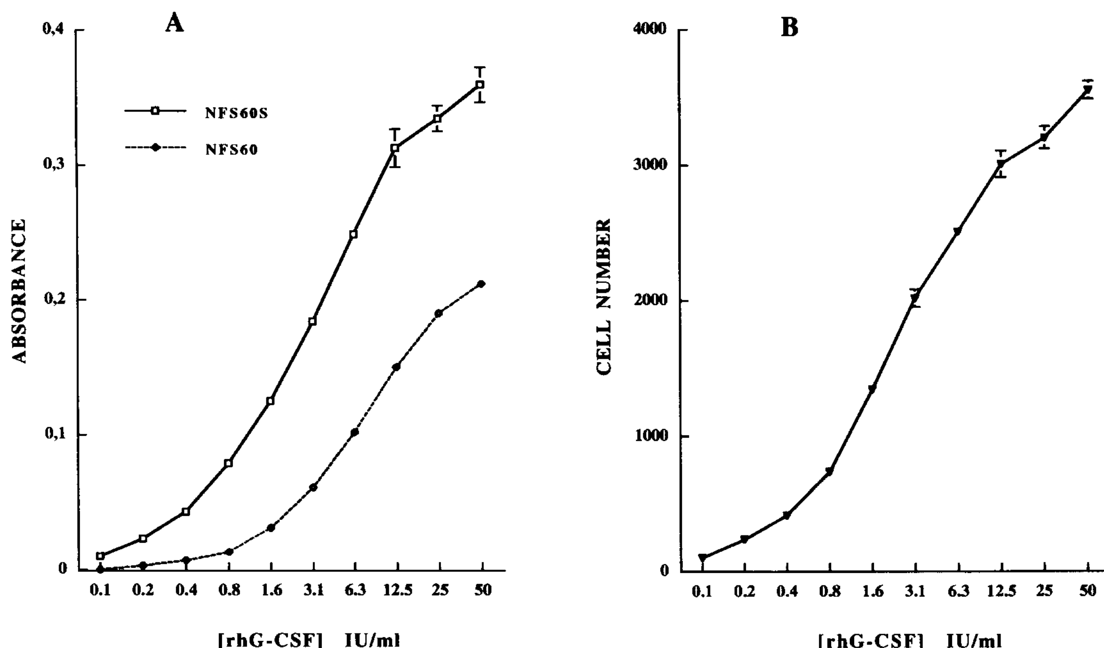

Figure 1
Proliferative stimulation of NFS-60 and NFS-60S cells by exposure to rhG-CSF. Growth was measured either by the MTT colorimetric microassay (A) or by determination of cell numbers using an electronic particle counter (B) as described in Materials and Methods. Curves in (A) represent the responses of the parental cell line (NFS-60) and the more sensitive subclone (NFS-60S) that was employed consistently as indicator. In (B), only results obtained from NFS-60S cells are shown. Each point shows the mean of five replicates and bars represent ±SEM. Cells were allowed to grow for 48 h, following addition of indicated concentrations of sargrastim, before addition of MTT solution and subsequent arrest of the enzymatic reaction 4 h later (A) or 52 h preceding counting (B). Absorbance values were calculated as outlined in Materials and Methods. The ordinate in (B) represents cell density × 1/100.

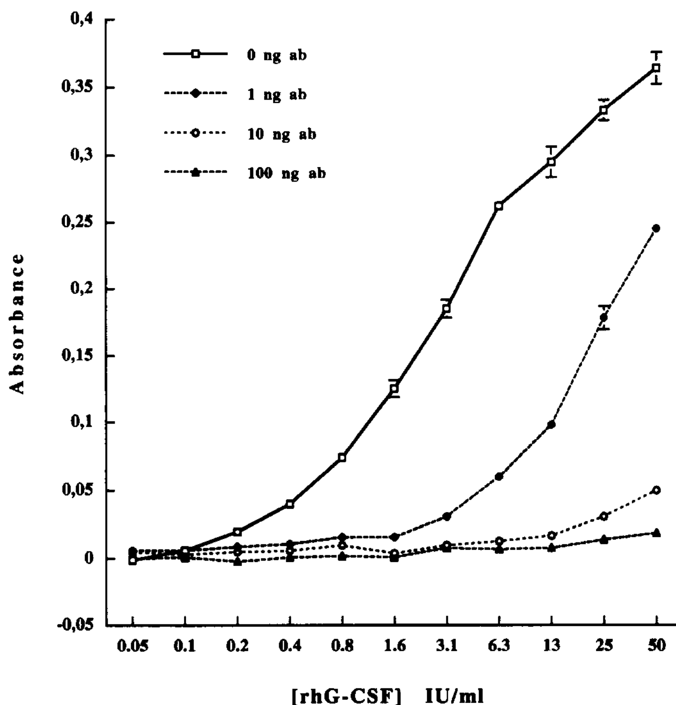

Figure 2
Neutralization of the NFS-60S response by monoclonal antibodies against hG-CSF. Bioactivity of rhG-CSF (sargrastim) was measured in the MTT colorimetric NFS-60S assay. The cytokine was either untreated or preincubated with indicated concentrations of antibodies (neutralizing anti hG-CSF). Vertical bars denote ±SED. The highest antibody titer employed, equalled the calculated hG-CSF-quenching activity in microwells containing the least diluted cytokine solution.

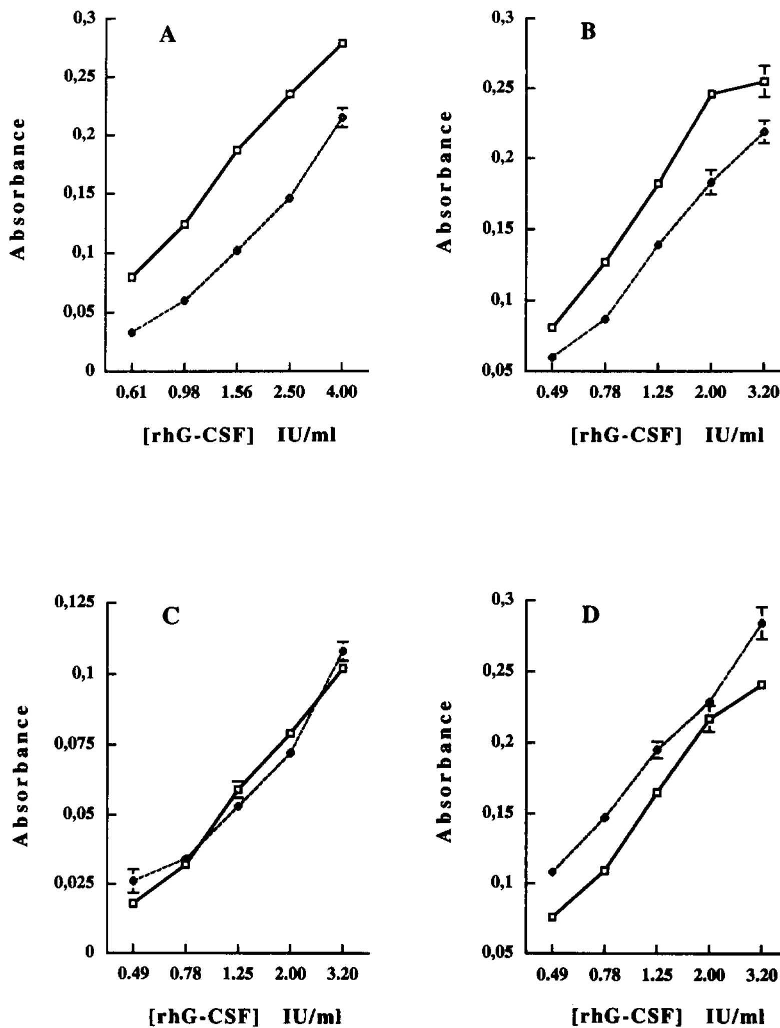

Figure 3
Potency determinations of rhG-CSF. Quantification of rhG-CSF with the NFS-60S bioassay, using the WHO 88/502 rhG-CSF preparation as a proliferative stimulator. Responses were obtained by MTT microcolorimetric assay. Vertical bars indicate ±SEM. Hatched lines represent test preparations that were diluted to fractions of the reference. (A) The test solution was diluted to 50% of the reference concentration ($T = 0.5R$); (B) $T = 0.7R$; (C) $T = R$; (D) $T = 1.3R$.

described above, employing concentrations of rhG-CSF, within the linear range of the stimulatory response curve. As outlined in Table 3, the obtained potencies were consistent within each assay cluster. The RSD values were low (7.9–12.0%). Notably, one of these sets (RSD = 12.0%) was subjected to both inter-analyst and inter-standard variation (two separate ampoules of the WHO 88/502 International Standard for rhG-CSF). Moreover, small differences were observed when potencies within each series, expressed as arithmetic mean values and as weighted combinations, respectively, were compared (Table 4). One of the formulations tested (Table 3, preparation 1) was produced in *E.coli* and hence devoid of carbohydrate modification. The stability of deglycosylated rhG-CSF has been reported to decrease substantially in the pH range 6–8, while the native-type cytokine is essentially unaffected by such changes [33]. Thus, we were prompted to investigate a possible influence to pH on the tested rhG-CSF preparations. For this purpose, the assay protocol was modified by an adjustment of our medium (employed for both sample dilution and cellular stimulation) to pH 6.6. As shown in Tables 3 and 4, this change significantly altered the measured potency of *E.coli*-derived rhG-CSF (filgrastim), to values that were accordant with the stated potencies. These results were roughly consistent with determinations based on medium titrated to pH 7.0, except that potency readings obtained with neutral pH

Table 1
Estimated potencies of four different dilutions of rhG-CSF as determined by the micro-colorimetric assay*

Potency (%)		Limits of error			
Nominal	Estimated	(%) $P = 0.95$	Statistical weight	Index of precision	RSD
50	52	93–108	4019	0.033	—
50	54	92–109	3221	0.048	—
50	49	91–110	2706	0.038	—
70	72	91–110	2471	0.061	—
70	63	94–106	6153	0.037	—
70	69	93–107	4593	0.044	—
70	65	90–111	2086	0.052	—
70	71	89–113	1569	0.076	—
70	65	93–107	4862	0.034	0.052
100	98	96–105	11773	0.025	—
100	100	95–105	10148	0.031	—
100	82	93–108	4437	0.040	—
100	93	93–108	4429	0.041	—
100	93	93–107	4785	0.039	—
100	111	89–112	1729	0.065	0.097
130	115	94–107	5782	0.035	—
130	124	96–105	11562	0.025	—
130	128	91–110	2812	0.049	—
130	138	86–116	1002	0.080	0.075

* Definitions of statistical weight, index of precision and RSD are specified in materials and methods.

Table 2
Weighted combinations of potency estimates presented in Table 1

	Estimated potency		Limits of error		Number of comb.
Nominal potency	Arithm. mean	Comb. result	(%) $P = 0.95$	Statistical weight	experim.
50	51	52	96–105	9901	3 (3)
70	68	67	97–103	20080	6 (6)
100	96	97	97–103	31153	4 (6)
130	126	125	96–104	15385	3 (4)

tended to be more variable (not shown). However, the activity of rhG-CSF, produced in Chinese Hamster Ovary (CHO) cells (lenograstim), was constant throughout these test conditions (Tables 3, 4). Dilution of filgrastim in modified assay medium (pH 6.6), before transfer to microwell cell cultures at physiological pH, resulted in only partially recovered potency readings (not shown). Thus, upon comparison of glycosylated G-CSF products with those devoid of carbohydrate, the deteriorating effect of alkaline environment on the latter type of substances should be considered.

Electrophoretic analysis of rhG-CSF

To further characterize the pH-dependent changes of filgrastim samples of this cytokine were subjected to SDS-PAGE and identification by Western detection, following incubation in assay medium at various times. Upon separation in the presence of 2-mercaptoethanol only minor dissimilarities were observed between samples exposed to various pH-conditions (not shown). However, when separated under non-reducing conditions a considerable decrease of rhG-CSF monomer became evident (Fig. 4a). In addition, several high molecular weight bands were resolved. Notably, two bands with MW around 70 kD and 100 kD could possibly represent tetra- and pentamer oligomers of rhG-CSF, respectively. A measure of monomeric filgrastim was obtained by densitometric readings of exposed X-ray films. Mean values from two separate experiments disclosed an appreciable reduction after 12 h at pH 7.4. This pattern became more pronounced by 36 h, whereas, lenograstim retained most of its material unpolymerized throughout the treatment (Table 5).

Table 3
Estimated potencies of two different preparations of rhG-CSF, including two separate batches (A and B) of these formulations, respectively

Preparation	A.P.*	Potency (%)†	Limits of error (%) P = 0.95	Statistical weight	Index of precision	RSD
IA	S	61	93–108	4084	0.049	—
		74	93–107	4734	0.043	—
		57	92–108	3713	0.041	—
		70	94–107	6461	0.031	—
		73	92–109	3046	0.056	—
		76	87–115	1147	0.077	—
		81	93–107	4486	0.038	0.120
IB	S	56	91–110	2270	0.058	—
		64	91–110	2351	0.049	—
		59	91–110	2452	0.055	—
IIA	S	83	89–112	1695	0.076	—
		83	94–107	5406	0.036	—
IIB	S	98	95–106	7285	0.037	—
		104	93–107	4780	0.046	—
IA	M	96	89–112	1779	0.075	—
		109	75–134	273	0.165	—
		92	89–113	1601	0.079	—
		94	87–115	1160	0.093	0.079
IB	M	104	92–109	3359	0.055	
		111	93–108	3954	0.050	
		102	93–107	4817	0.046	
		122	91–110	2343	0.064	0.082
IIA	M	69	86–116	1024	0.093	
		84	87–114	1300	0.086	
IIB	M	103	88–113	1484	0.071	
		93	84–119	770	0.114	

*Assay protocol; standard (S; pH 7.4) and modified (M; pH 6.6) protocol (see Materials and Methods).
†Defined here as percentage of WHO 88/502 activity. Definitions of statistical weight, index of precision and RSD are specified in Materials and Methods.

Table 4
Weighted combinations of potency determinations shown in Table 3

Preparation	A.P.†	Estimated potency*		Limits of error (%) P = 0.95	Statistical weight	Number of comb. exp.
		Arithm. mean	Comb. result			
IA	S	70	71	96–104	15385	4 (7)
IB	S	60	60	95–106	7042	3 (3)
IIA	S	83	83	95–106	7092	2 (2)
IIB	S	101	100	96–104	12077	2 (2)
IA	M	98	95	94–107	4808	4 (4)
IB	M	110	106	96–104	12121	3 (4)
IIA	M	77	81	90–111	1908	2 (2)
IIB	M	98	99	91–110	2252	2 (2)

*Percentage potency of WHO 88/502.
†Assay protocol; standard (S; pH 7.4) or modified (M; pH 6.6) assay medium (see Materials and Methods).

Analysis of partially heat-inactivated rhG-CSF

Finally, rhG-CSF, subjected to an incubation at 60°C for 2 h, was assayed in the NFS-60S system. The untreated preparation was employed as a reference for potency. An approximate 35% decay was observed in the treated material (Table 4). When samples of both types were analysed by SDS-PAGE/ Western detection, in the presence of β-mercaptoethanol, a very faint band of 70–72 kD was occasionally observed together with the heat-treated G-CSF monomer (Fig. 4b). The difference was more evident under non-reducing conditions with a slow-migrating species of about 100 kD observed in the lane containing treated cytokine (Fig. 4b). This change was paralleled by a reduction of intensity in the monomer band. rhG-CSF with various degrees of O-linked carbohydrate modification, i.e. expressed in *S. cerevisiae*

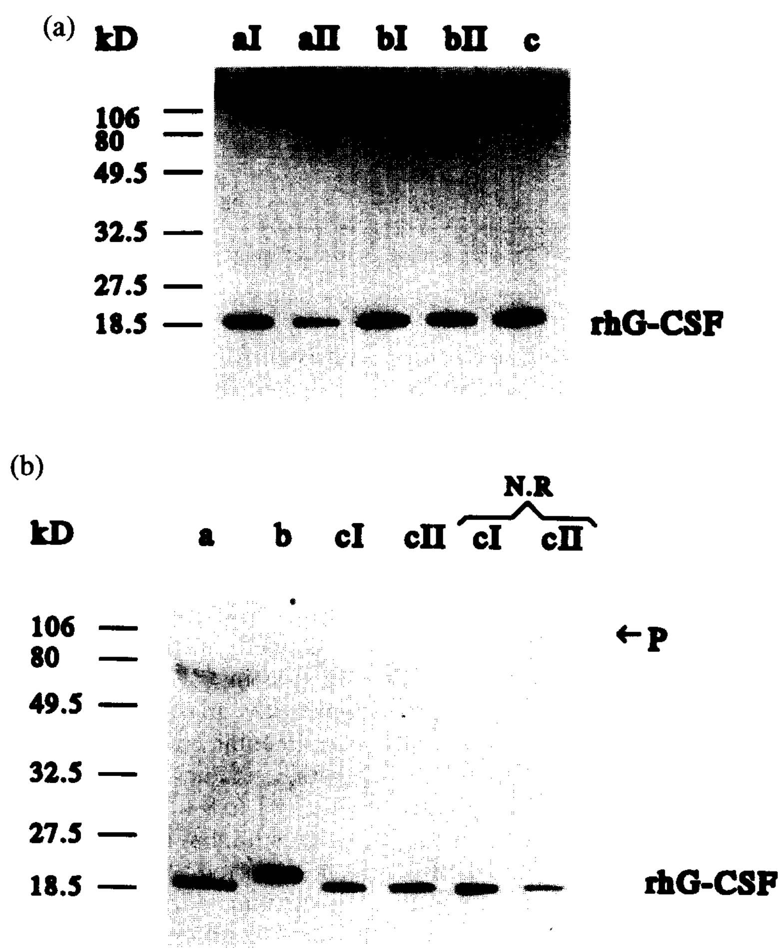

Figure 4
Western immunoblot of rhG-CSF. SDS-PAGE, electrotransfer and development techniques were conducted as outlined in Materials and Methods. Approximately 8 ng of rhG-CSF was loaded in each lane. (A) aI and aII indicate samples incubated for 12 and 36 h, respectively, in standard assay medium (pH 7.4). bI and bII denote the same material treated accordingly in modified assay medium (pH 6.6). c represents untreated filgrastrim. (B) Lane a: WHO 88/502; lane b: CHO-derived substance. Lanes cI and cII: *E. coli*-derived rhG-CSF, untreated and partially inactivated protein, respectively. N.R. indicates samples separated under non-reducing conditions. P indicates the high-MW G-CSF band. The sample in lane a has a high content of human serum albumin, which is a likely identity of the band at around MW 68 kD.

Table 5
Densitometric readings of *E. coli*-derived monomeric rhG-CSF* following incubation in standard (S; pH 7.4) and modified (M; pH 6.6) assay medium at indicated times

Assay medium	Incubation time (h)	I.O.D. (%)†
S	12	63
S	36	31
M	12	94
M	36	84

*Separated by SDS-PAGE under non-reducing conditions (see Materials and Methods).
†Percentage of untreated sample (I.O.D. = integrated optic density); arithmetic mean of two separate experiments.

(International Standard) and in CHO cells, respectively, were used as reference substances.

Discussion

We have employed the murine myeloid leukemia cell line NFS-60 to establish the clone NFS-60S, which displayed a linear growth stimulation at lower concentrations of rhG-CSF than the parental line. The assay described is based on a colorimetric detection system which permits reliable analysis of pro-

Table 6
Potency readings of a partially inactivated preparation of rhG-CSF

Preparation	Potency (%)*	Limits of error (%) $P = 0.95$	Statistical weight	Index of precision
IA; 60° C × 2 h	71	88–113	1416	0.0800
	62	91–110	2415	0.0472

*Percentage activity of untreated rhG-CSF preparation. Statistical weight and index of precision are explained in Materials and Methods.

liferative stimulation without the need for radionuclides. The specificity of the system was demonstrated in tests for stimulation, using a panel of substances, including molecules that are functionally related to hG-CSF. Among those, only rhIL-6 induced a detectable but weak effect, at comparatively high concentrations. Moreover, the cellular response to the rhG-CSF could be entirely neutralized by antibodies against this cytokine.

The NFS-60 cell line has been reported as sensitive to both IL-3 and IL-6 of murine origin [30, 34]. However, with a view to the structural divergence between human and murine IL-3 [35, 36], the lack of response of NFS-60S cells to the species-heterologous cytokine is an expected finding. Growth-stimulation by hIL-6, which is more conserved between these species [37], has previously been documented [38]. Candidate explanations for the relatively limited hIL-6 dependent response, showed by the NFS-60S clone, are alterations in receptor expression or in the signal transduction machinery as a consequence of the prolonged maintenance in rhG-CSF-supplemented culture medium.

The assay was further evaluated in two separate types of experiments: assessment of potencies in solutions of rhG-CSF diluted to known concentrations and determination of samples with unknown activity. In both types of analyses, individual assays generally displayed high precision, indicated by a low index of precision, and narrow limits of error. Moreover, the potency readings were reproducible, which also was suggested by the large number of estimates that could be used for combination [26].

The recently adopted International Standard for Human Recombinant G-CSF (WHO 88/502) is based on sargrastim (yeast-derived rhG-CSF) [22]. This preparation was employed in our potency determinations as a reference for G-CSF bioactivity. Low levels of filgrastrim (*E.coli*-derived rhG-CSF) bioactivity were consistently obtained when the assay was conducted at physiological pH. However, after the introduction of a slight modification, i.e. pH-shift to 6.6, the measured activities were restored to stated potency levels. These findings were supported by the appearance of several high molecular weight bands, with a concomitant diminution of monomer, in Western/SDS-PAGE analysis of filgrastim, maintained at pH 7.4 for various periods. Lenograstim (CHO-derived rhG-CSF) was apparently unaffected by such differences in experimental conditions, as judged from our bioassay studies. A pH-dependence in the range pH 6–8 in bioactivity of deglycosylated lenograstim, but not the intact counterpart, has been reported. Polymerization of the sugar-free cytokine parallels the rapid inactivation in alkaline solutions [33]. In terms of carbohydrate modification, the trimmed lenograstim molecule is identical with filgrastim. Hence, our results compare favourably with previous findings. Determination of biomolecular activity would regularly require identical substances as standards for potency. For example, subtle dissimilarities between various subtypes of human interferon-alpha have resulted in the establishment of a panel of cognate standard preparations [39]. In this study, however, we have evaluated a slightly modified assay protocol that circumvents evident problems with the utilization of the present International Standard for Human Recombinant G-CSF when reading potencies of filgrastim. Furthermore, sargrastim, with an incomplete carbohydrate structure, appeared relatively resistant to pH changes in our hands and thus resembled the lenograstim-type of rhG-CSF. Hence, the existence of glycosylation at the correct position, but not necessarily of native complexity, appears to be a requisite for protection against polymerization of rhG-CSF in neutral and mildly alkaline solutions.

The optimal range for quantitative analysis of rhG-CSF was about 4–60 pg ml^{-1}, corre-

sponding to almost one magnitude higher sensitivity than that of the reported immunoassay systems for this cytokine [40, 41]. In addition, bioassays have the advantage of verifying biological activity of measured molecules. This feature should be valuable when determining potencies of recombinant polypeptides with variable specific activities, due to small differences in primary structure or in carbohydrate modification.

Biological assay systems for hG-CSF have been described by others [28, 42–45]. However, none of these methods has been designed to fulfil pharmacopoeial requirements for the quantification of pharmaceutical cytokine preparations. The presented bioassay measures native hG-CSF as well as deglycosylated rhG-CSF, produced in *E.coli*, with high precision and reliability and should, therefore, be a candidate as a pharmacopoeial method for potency assessments of preparations of this substance. Moreover, it is fairly easy to perform and is accomplished within 72 h and would thus be useful for other purposes.

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