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Application of the CFU-GM Assay to Predict Acute Drug-Induced Neutropenia: An International Blind Trial to Validate a Prediction Model for the Maximum Tolerated Dose (MTD) of Myelosuppressive Xenobiotics

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Fn1 **In a previous study of prevalidation, a standard operating procedure (SOP) for two independent *in vitro* tests (human and mouse) had been developed, to evaluate the potential hematotoxicity of xenobiotics from their direct and the adverse effects on granulocyte-macrophages (CFU-GM). A predictive model to calculate the human maximum tolerated dose (MTD) was set up, by adjusting a mouse-derived MTD for the differential interspecies sensitivity. In this paper, we describe an international blind trial designed to apply this model to the clinical neutropenia, by testing 20 drugs, including 14 antineoplastics (Cytosar-U, 5-Fluorouracil, Myleran, Thioguanine, Fludarabine, Bleomycin, Methotrexate, Gemcitabine, Carmustine, Etoposide, Teniposide, Cytoxan, Taxol, Adriamycin); two antivirals (Retrovir, Zovirax,); three drugs for other therapeutic indications (Cyclosporin, Thorazine, Indocin); and one pesticide (Lindane). The results confirmed that the SOP developed generates reproducible IC90 values with both human and murine GM-CFU. For 10 drugs (Adriamycin, Bleomycin, Etoposide, Fludarabine, 5-Fluorouracil, Myleran, Taxol, Teniposide, Thioguanine, and Thorazine), IC90 values were found within the range of the actual drug doses tested (defined as the actual IC90). For the other 10 drugs (Carmustine, Cyclosporin, Cytosar-U, Cytoxan, Gemcitabine, Indocin, Lindane, Methotrexate, Retrovir, and Zovirax) extrapolation on the regression curve out of the range of the actual doses tested was required to derive IC90 values (extrapolated IC90). The model correctly predicted the human MTD for 10 drugs out of 10 that had "actual IC90 values" and 7 drugs out of 10 for those having only an extrapolated IC90. Two of the incorrect predictions (Gemcitabine and Zovirax) were within 6-fold of the correct MTD, instead of the 4-fold range required by the model, whereas the prediction with Cytosar-U was approximately 10-fold in error. A possible explanation for the failure in the prediction of these three drugs, which are pyrimidine analogs, is discussed. We concluded that our model correctly predicted the human MTD for 20 drugs out of 23, since the other three drugs (Topotecan, PZA, and Flavopiridol) were tested in the prevalidation study. The high percentage of predicitivity (87%), as well as the reproducibility of the SOP testing, confirm that the model can be considered scientifically validated in this study, suggesting promising applications to other areas of research in developing validated hematotoxicological *in vitro* methods.**

Key Words: GM-CFU assay; acute neutropenia; maximum tolerated dose; phase I trial; myelotoxicity.

In vitro models of hematopoiesis are being used increasingly in investigative hematopathology and in preclinical safety studies on candidate drugs (Deldar, 1994; Deldar and Parchment, 1997; Deldar and Stevens, 1993). These models are also useful for determining the relative sensitivities of various animal species to haematotoxic effects and for studying synergistic and antagonistic effects of several compounds (Du *et al.*, 1990). The type of hematotoxicity most frequently and most thoroughly studied *in vitro* is the acute effect of toxicants on bone marrow progenitors, such as granulocyte-macrophages (CFU-GM), erythroids (CFU-E), and megacaryocytes (CFU-MK), which is quantified from the number of surviving progenitors as a function of exposure level under maximally stimulatory cytokine concentrations (Metcalf, 1984). Since haematotoxicity can result from either the direct interference of the toxicant with the different haematopoietic progenitors or with the expression of cytokines and their receptors, many different protocols have been developed and proposed for *in vitro* hematotoxicity testing (Gribaldo *et al.*, 1996, 1998; Lewis *et al.*, 1996; Naughton *et al.*, 1992; Noble and Sina, 1993; Parchment *et al.*, 1998; Parent-Massin and Thouvenot, 1995; Parent-Massin *et al.*, 1993; Pessina, 1998; Pessina *et al.*, 1999; San Roman *et al.*, 1994; Schoeters *et al.*, 1995; Van Den Heuvel *et al.*, 1997). All these tests are modifications of the original technique suggested by Bradley and Metcalf (1966)

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and then developed and modified by other authors (Dexter and Spooncer, 1987; Dexter and Testa, 1976; Dexter *et al.*, 1973). *In vivo* substances such as antineoplastics, microbial toxins, and ionizing radiation (Bruce *et al.*, 1966; Grande and Bueren, 1995) destroy the rapidly dividing marrow progenitors, and a single exposure can result in acute, reversible neutropenia or thrombocytopenia 4 to 20 days later. A rapid repopulation of the progenitor compartment precedes the recovery of peripheral counts by several days (Greenberg *et al.*, 1974; Neelis *et al.*, 1997). Important goals during preclinical drug development is to predict whether a new agent will be clinically toxic to the bone marrow, whether the toxicity will be specific to one cell lineage (lymphocytes, neutrophils, megakaryocytes, or erythrocytes), at what dose or plasma level the drug will be toxic, which model best predicts the clinical situation, and when the onset and nadir of cytopenia and the onset of recovery will be likely to occur. Myelotoxicity is one of the major limitations to the use of full doses of antitumor agents, and the goal in the regulatory setting usually emphasizes the prediction of two levels of exposure: the highest dose that will not cause a clinically adverse effect and the dose that causes maximally tolerated, reversible perturbations in peripheral blood counts, termed the maximum tolerated dose (MTD). Selection of the starting dose for the phase I trial, which is typically based on the MTD of the most sensitive species, is critical, in that the dose selected must not be toxic, while at the same time it must be high enough to give the patients therapeutic benefit. Even with the advent of the use of molecular targeting to develop new therapeutic approaches, the majority (>50% in the last 15 years) of anticancer drugs still produce myelosuppression as the dose-limiting toxicity (DLT) in humans (Parchment, 2000; Parchment *et al.*, 1998).

In vitro tests could refine safety margins by reducing toxicological uncertainties due to animal/human extrapolation, and would provide a more rational basis for calculating clinical dosages and for setting human exposure limits. With anticancer drugs, *in vitro* studies should be undertaken to identify those compounds that are significantly more toxic to humans than to either dogs or rodents. By identifying such compounds, it would be possible to decrease the risk of a lethal overdose in the first cohort of patients to which they are administrated, a risk that cannot be identified during current preclinical testing strategies. An *in vitro* assay could highlight the potency difference between humans and the preclinical test species, so that the starting dose in phase I clinical trials could be considerably closer to the MTD, without compromising safety. Thus, not only would phase I clinical trials be completed more quickly, but fewer patients would be treated with ineffective doses. In this respect, the predictivity of the data obtained from animal studies could be increased by *in vitro* tests, and the level of uncertainty concerning human safety could be decreased (Grande and Bueren, 1995). Validated *in vitro* tests for hematotoxicity could help to answer some of the above-mentioned questions, and contribute to a reduction in the number of animals required in preclinical toxicology (Balls *et al.*, 1995; Curren *et al.*, 1995).

As reported in Parchment *et al.* (1994), a correlation was found between the severity of neutropenia in the clinic with pyrazoloacridine and the inhibition of CFU-GM *in vitro*. Subsequently, *in vitro/in vivo* correlations were found for the camptothecins (Erickson-Miller *et al.*, 1997) and anguidine (Parent Massin and Parchment, 1998). A key finding in these studies was that the concentration that inhibited CFU-GM by 90% (IC90) was a more predictive endpoint for the MTD in animals and in humans than the IC50 (Parchment *et al.*, 1997, 1998). The success to date lies primarily in the identification of the *in vitro* inhibition concentration that can predict the MTD. In a previous prevalidation study (on six anticancer drugs) a standard operating procedure (SOP) for murine and human CFU-GM was developed. Reproducible IC90 values obtained with SOP were used to predict human MTDs, which were compared to the actual human MTD (Pessina *et al.*, 2001). In this paper, we describe an international validation study supported by the European Centre for the Validation of Alternative Methods (ECVAM) designed to evaluate the predictive capacity of this model when applied to clinical neutropenia, by testing an additional 20 drugs. The prediction model adopted utilizes information from the *in vitro* analysis of toxic effects on the actual human target cell, and offers the advantage of being mechanism-naïve and would only fail to identify hematotoxicants that adversely affect myelopoiesis via indirect physiological mechanisms such as induced release of inhibitory cytokines, inhibited release of stimulatory cytokines, or metabolic activation of pro-toxicants. It provides human toxicology and pharmacologic information in the laboratory setting and an experimental basis for selecting the best animal models for investigating clinical haematotoxicity.

MATERIALS AND METHODS

Methylcellulose Culture Medium

The medium for murine and human cells was prepared by StemCell Technologies (Vancouver, BC, Canada) according to specific modifications of the Methocult, as suggested by the management team of the validation study (Pessina *et al.*, 2001). The medium contained 1% methylcellulose in IMDM (Iscove's Modified Dulbecco's Medium), 30% fetal bovine serum (FBS), 1% bovine serum albumin, 2- mM L-glutamine, and 10-ng/ml granulocyte/macrophage-colony stimulating factor (human-rec-GM-CSF or murine-rec-GM-CSF). A unique batch of each medium was prepared, and aliquots of the same batch were supplied frozen to the laboratories.

Haematopoietic Progenitors

Human umbilical cord blood cells (hu-CBC) from five different donors were supplied frozen by Poietic Technologies (Gaithersburg, MD), according to a protocol approved by the Institutional Review Board (IRB), and all of the cryotubes were stored in liquid nitrogen. A sufficient number of aliquots of each CBC donor was distributed to each laboratory, so that all participants could work on the same batch of a cell preparation during each experimental phase.

Murine bone marrow cells were collected from male BDF/1 (C57Bl/

6×DBA-2) mice, 8–12 weeks old, according to the procedure described in Pessina *et al.* (2001).

Drug Selection

Twenty drugs were selected according to their recognized or potential hematotoxicity in clinical use. As negative controls, drugs with no previously described hematotoxicity were selected. The main characteristics of the drugs selected are reported in Table 1. The list comprised 14 antineoplastics (Cytosar-U, 5-Fluorouracil, Myleran, Thioguanine, Fludarabine PO_4, Bleomycin, Methotrexate, Gemcitabine, Carmustine, Etoposide, Teniposide, Cytoxan, Taxol, Adriamycin); two antivirals (Retrovir and Zovirax,); three other therapeutic indications (Cyclosporin, Thorazine, and Indocin); and one pesticide (Lindane).

The drugs were purchased from Washington Wholesale Drug Exchange (Savage, MD) and were then prepared, coded, and distributed under GLP conditions by an independent laboratory (Charles K. Grieshaber, Oncology Research and Regulatory Consulting Associates, Grosse Pointe Woods). Each drug and solvent were assigned a code number, unique for each participanting laboratory, to guarantee the rigorous "blind" conditions of the study.

Experimental Design

To evaluate the intralaboratory and interlaboratory variabilities of the in vitro assay, three laboratories (Brest, Milan, Mol) tested the drugs on the murine model (40,000 mu-BMC/dish), and three laboratories (Detroit, Ispra, Madrid) used the human model (75,000 hu CBC/dish).

The experimental design was optimized for testing three drugs (in triplicate) simultaneously on one day for each experiment. Each drug was tested three times: The first experiment was a screening test (ST) to determine the range of the drug's activity; the other two tests were performed to determine the inhibitory concentration (IC) by using a narrower range of drug concentrations as described below. For each experiment an internal control was set up to confirm the linear relationship between the cells seeded and the colonies scored. This control was based only on the assessment of two points (CTRL1 and CTRL3) of the curve studied in the prevalidation phase and corresponded to 2,500 and 40,000 cells/dish for the murine model and to 10,000 and 75,000 cells/dish for the human model (Pessina *et al.*, 2001).

CFU-GM Assay

The detailed SOP was given in the report of the prevalidation study (Pessina *et al.*, 2001). Here we describe only the modifications concerning drug dilution and the preparation of tubes, which were changed as a consequence of the "blind conditions" needed to perform the validation study. Briefly, 11 tubes of cell culture mixture were prepared for each experiment, according to the following experimental design: CTRL1 and CTRL3 (linearity controls), D_0 (vehicle controls), and D_1–D_8 for the dose–response curve. To each tube containing 4.0 ml of methylcellulose culture medium were added 100 μl of IMDM (to CTRL1 and CTRL2) and 78 μl of IMDM (to D_0–D_8). Then, 22 μl of the vehicle were added to D_0 and 22 μl of each toxicant dilution to D_1–D_8. Each tube was used to prepare three culture dishes. All of the toxicant dilutions were prepared at ×200 the final dilution, in order to obtain the final fold dilutions of drug in the culture dish of 5×10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} from D_8 to D_1, respectively (the concentration of the vehicle was of 0.5% vol for each dilution).

The cultures were incubated at 37°C in air + 5% CO_2 under saturated humidity for 7 days (murine assays) or 14 days (human assays). All dishes were scored for colony counts in a random fashion. The different types of colonies (compact colonies, diffuse and spread colonies, multicentric colonies, burst-forming units) were all counted as one colony. Since the humidity level is a critical parameter during incubation, the evaporation rate (ER) was determined as described in Pessina *et al.* (2001).

Passing from the Screening Test to IC Determination Phase

The GFU-GM results obtained in the Screening Test enabled the following to be identified: the first drug dilution that completely inhibited CFU-GM (FCID) and the last drug dilution that did *not* inhibit CFU-GM (LNID). Then the log dose differential χ between the LNID and FCID was calculated, as follows:

$$\chi = \log\{\text{final toxicant dilution @ LNID}\} - \log\{\text{final toxicant dilution @ FCID}\}.$$

To determine the new drug concentrations to use in the two assays of the IC determination phase, the FCID was assigned to D_8 and the LNID to D_2, the χ value was divided into six parts of size ($\phi = \chi$: 6), and these parts were assigned to the dilution levels D_n (D_2–D_7) according the following formula:

$$D_n = D_{LNID} \times 10^{-(n-2)\Phi}$$

In order to reduce the variability, all of the dilutions were performed by starting from the D_8 concentration of toxicant stock. However, under some conditions, the volume required for making the dilution was smaller than could be pipetted, so, for these concentrations, dilutions of the drug stock were prepared before making the working stock.

Statistical Analysis

At the end of the study, the data were collected by each laboratory in an Excel template, the test drugs were decoded, and the actual concentrations used in the assay were calculated. The statistical analysis of the data was performed by means of the Generalized Linear Model and PROBIT procedures of the SAS package (SAS Institute Inc., Cary, NC). For all of the comparisons, a 0.05 α-type error was considered as significant. Three main steps were followed:

Verifying the linear relationship: Cells seeded-CFU-GM colonies. The slope of the line between two internal controls (CTRL1 and CTRL3) was estimated by considering the ratios between CTRL3 and CTRL1 and then testing the null hypothesis that the slope was equal to zero. The homogeneity of results for each laboratory (and experiment) was verified by performing an analysis of variance (ANOVA). The percentage of coefficient of variability (CV %) at CTRL3 (calculated as standard deviation/mean × 100) was also considered.

Analyzing the best fitting model. Several regression models were fitted to study the inhibition of CFU-GM in IC determination experiments. A goodness of fit test, based on the likelihood ratio, chi-square, was verified for each drug and for each species tested (human or murine), according to the drug concentration (expressed as μg/ml), and three cumulative distribution functions were used to model the response probabilities (NORMAL: normal distribution for the probit model; LOGISTIC: logistic distribution for the logit model; GOMPERTZ: Gompertz distribution for the gompit model). For each species (murine and human), drug, and laboratory, the best fitting model was selected and the sources of variability were identified for the whole curve.

Estimating the inhibitory concentrations. Based on the best fitting model selected for each drug, the ICs were estimated from IC1 to IC99 by the probit procedure. Homogeneity of the inhibitory concentrations was analysed by studying both the interlaboratory and the intralaboratory variability by ANOVA.

Prediction Model

The prediction model for calculating the human MTD was based on the following algorithm:

TABLE 1
Main Features of Drugs Selected for the Validation Study

Drug	MW	Therapy indication	Main recognized mechanisms of action	Main evidenced clinical toxicity
Adriamycin (Doxorubicin)	543.53	Antineoplastic	Inhibition of topoisomerase II Activation of protein kinase C mediated signal trasduction pathway Free radical generation Stimulation of apoptosis	Myelosuppression Mucositis Cardiac toxicity Severe local tissue damage after drug extravasation
Bleomycin (Blenoxan)	$A_2 \sim 1{,}400$	Antineoplastic	Binding to DNA, which causes breaks in single and double strands Radical formation Inhibition of DNA synthesis	Anaphylactoid reactions Anorexia Blistering Hyperkeratosis of the palms Pulmonary fibrosis
Carmustine (BCNU)	214.1	Antineoplastic	Alkylation of DNA Cross-linking of DNA	Suppression of myelopoiesis
Cyclosporin	1202.6	Immunosuppressive	Binds to intracellular cyclophilin and inhibits T-cell function	Neurotoxicity Liver dinsfunction Unremarkable bone marrow toxicity
Cytosar-U (Cytarabine)	243.22	Antineoplastic	Inhibition of DNA polymerase	Myelosuppression Stomatitis Alopecia
Cytoxan (Cyclophosphamide)	279.1	Antineoplastic	Binding to DNA, which causes breaks in single and double strands Inhibition of DNA synthesis	Myelosuppression Hemorrhagic cystitis Alopecia
Etoposide	588.6	Antineoplastic	Inhibition of topoisomerase II Block of cell cycle S-G_2 phase DNA degradation Inhibition of nucleoside transport Inhibition of mitochondrial electron transport	Alopecia Significant toxicity to the hematopoietic and lymphoid system
Fludarabine	285.2	Antineoplastic	Inhibition of ribonucleotide reductase Inhibition of DNA synthesis	Myelosuppression Neurotoxicity
5-Flurouracil	130.1	Antineoplastic	Inhibition of cell cycle progression Inhibition of DNA synthesis	Neurotoxicity Oral and gastrointestinal ulceration Bone marrow depression
Gemcitabine (Gemzar)	299.66	Antineoplastic	Inhibition of cell cycle progression Inhibition of DNA synthesis	Myelosuppression Skin rash
Indocin (Indomethacin)	357.8	Anti-inflammatory	Inhibition of prostaglandin synthesis	Depression Pancreatitis Gastrointestinal hemorrhage Thrombocytopenia Aplastic anemia
Lindane	290.85	Insecticide	Modification of Na^+ and K^+ permeability Block of nervous transmission	Neurotoxicity Hematotoxicity Mutagenicity
Methotrexate	454.5	Antineoplastic	Inhibition of dihydrofolate reductase Arrest of DNA, RNA, and protein synthesis	Oral and gastrointestinal tract Ulceration Myelosuppression
Myleran (Busulfan)	246.3	Antineoplastic	Binding to DNA, which causes breaks in single and double strands Inhibition of DNA synthesis	Myelosuppression Hemorrhagic cystitis Alopecia
Retrovir (Zidovudine)	267.24	Antiviral	Inhibition of HIV reverse transcriptase	Myelosuppression
Taxol (Paclitaxel)	853.92	Antineoplastic	Inhibition of mitosis by stabilization of microtubules	Acute hypersensivity reaction Myelosuppression Mucositis
Teniposide	656.65	Antineoplastic	Block of cell cycle S-G_2 phase DNA degradation Inhibition of nucleoside transport Inhibition of mitochondrial electron transport Inhibition of topoisomerase II	Alopecia Myelosuppression Lymphopenia
Thioguanine	167.2	Antineoplastic	Block of cell cycle Inhibition DNA synthesis	Myelosuppression
Thorazine (Chlorpromazine)	318.8	Anti-emetic Antipsychotic	Block of dopaminergic receptors	Tardive dyskinesia Neuroleptic Malignant Syndrome
Zovirax (Acyclovir)	225.2	Antiviral	Inhibition of herpes virus DNA polymerase Inhibition of viral DNA replication	Diarrhea Myelosuppression at high doses

Note. References: Dorr and Von Hoff (1994) and Chabner and Longo (1996).

$$\text{Predicted Human MTD (P-HuMTD)} = \text{Actual Murine LD10} \times \frac{\text{Human IC90}}{\text{Murine IC90}} \text{ (in CFU-GM assay)}$$

This prediction model, studied and applied in the prevalidation study (Pessina *et al.*, 2001), correlates the inhibition of CFU-GM *in vitro* and the depth of the absolute neutrophil count (ANC) nadir *in vivo*. Because pharmacokinetic differences across species may contribute to as much as a 4-fold difference in MTD, and the prediction model does not accommodate this source of variability, we considered an "accurate prediction" as the prediction of a human MTD that lies within 4-fold of the actual human MTD value (Erickson-Miller, 1997; Parchment *et al.*, 1993, 1994; Volpe *et al.*, 1996).

RESULTS

Internal Linearity Control

The absolute counts of CFU-GM in CTRL3 controls were 81.7 ± 53.4 for human and 83.8 ± 46.6 for murine colonies. The ratios CTRL3 /CTRL1 gave means of 13.5 ± 0.9 for the murine and 9.2 ± 0.4 for the human model. The linearity and proportionality of response are independent of the absolute number of colonies that each laboratory obtained in D_0 dishes (which can differ greatly). Only one test out of 300 had to be repeated because the linearity test failed and produced unacceptable results (human model with CTRL3/CTRL1 ratios < 7).

The statistical analysis of the internal linearity controls verified the required linearity and proportionality between the number of cells seeded and colonies counted, confirming a good correlation with high coefficient of determination values of $R^2 = 0.45$ in the human model and $R^2 = 0.57$ in the murine model. No significant intralaboratory and interlaboratory variability was observed (human, $p = 0.60$; murine, $p = 0.18$), and the regression showed a very significant slope ($p = 0.0001$). On the basis of these results, all of the experiments performed were considered to meet the acceptance criteria, thereby confirming the good performance of the protocol observed previously in the prevalidation study.

IC Determination

For over 90% of the drugs, both in human and murine, the best fitting model was compatible with the normal or logistic distribution, and since the chi-square values did not differ significantly between them, the following analysis was performed by considering, for all of the drugs, the normal distribution. To normalize the dose–response curve, all of the regression lines were determined by considering as 100% the absolute counts of CFU-GM scored in D_0 (vehicle control).

T2-T3 As is shown in Tables 2 and 3, for some drugs, all of the IC values were determinable within the range of the actual concentrations tested, which were calculated by interpolation on the fitted regression curves. For the other drugs, the IC values had to be extrapolated out of the range of drug concentrations tested. For these drugs the reliability of the extrapolated IC values strongly depends on the maximal experimental IC actually determined by the assay. Therefore, the degree of reliability of the IC90 was predicted on the basis of the maximal common IC (McIC) that the test was able to estimate both in the murine and human models within the range of the actual drug concentrations in three laboratories (McIC > 50: acceptable reliability; McIC < 50: poor reliability).

Human model. As is shown in Table 2, for 15 drugs (Adriamycin, Bleomycin, Carmustine, Cytosar-U, Cytoxan, Etoposide, Fludarabine, 5-Fluorouracil, Gemcitabine, Myleran, Retrovir, Taxol, Teniposide, Thioguanine, Thorazine), the "actual IC50 value" was interpolated on the curve by all of the laboratories while for Methotrexate it was interpolated by only one laboratory. For Cyclosporine, Indocin, Lindane, and Zovirax, the IC50 value was not determinable in any laboratory.

The IC90 value was interpolated, within the range of doses tested, for 10 drugs (Adriamycin, Bleomycin, Etoposide, Fludarabine, 5-Fluorouracil, Myleran, Taxol, Teniposide, Thioguanine, and Thorazine) by three laboratories, for two drugs (Cytoxan and Gemcitabine) by two laboratories, for two drugs (Cytosar-U and Retrovir) by one laboratory. For the remaining six drugs (Indocin, Lindane, Methotrexate, Zovirax, Carmustine, and Cyclosporin), the IC90 could not be estimated. Based on the McIC value determinated in each laboratory, the reliability of the IC90 values extrapolated from the regression curve was acceptable for five drugs (Carmustine, Cytosar-U, Cytoxan, Gemcitabine, and Retrovir), since their McIC > IC50. For the other drugs the extrapolation of IC90 values is likely to be imprecise, because the McIC was lower than the IC50.

The ANOVA showed a high intralaboratory homogeneity (only Adriamycin for IC50 values showed a $p < 0.05$). A greater heterogeneity for four drugs was observed for interlaboratory variability: IC50 (Adriamycin and Methotrexate) and IC50 and IC90 (Myleran and Taxol). However, this significant interlaboratory variation affected the IC90 values, and therefore the prediction model, for only two drugs.

These results clearly indicate that, in the determination of IC, the laboratory represented a significant source of variability for 4 drugs out of 20 (corresponding to 20% of the drugs tested) and for 6 IC determinations out of 40 (15%).

Murine model. The analysis of data from the murine model showed a high degree of homogeneity (Table 3). For 15 drugs (Adriamycin, Bleomycin, Cyclosporin, Cytosar-U, Etoposide, Fludarabine, 5-Fluorouracil, Gemcitabine, Methotrexate Myleran, Taxol, Teniposide, Thioguanine, Thorazine, and Zovirax), the IC50 values were determinable by three laboratories and for one drug (Carmustine) by two laboratories. For three drugs (Indocin, Lindane, and Retrovir), IC50 was not estimable by any laboratory.

The IC90 values were determinable by three laboratories for 12 drugs (Adriamycin, Bleomycin, Cytosar-U, Etoposide, Fludarabine, 5-Fluorouracil, Gemcitabine, Myleran, Taxol, Teniposide, Thioguanine, and Thorazine), by two laboratories for

TABLE 2
IC50 and IC90 Values (Expressed as μg/ml) Determined in Human Model

Drug	IC value	Mean ± SD	*p* values[a]	
			Interlaboratory	Intralaboratory/interexperiment
Adriamycin	IC50	0.0047 ± 0.0001	0.0009*	0.004*
	IC90	0.01 ± 0.0005	0.062	0.160
Bleomycin	IC50	0.94 ± 0.24	0.726	0.632
	IC90	2.28 ± 0.28	0.355	0.704
Carmustine	IC50	12.13 ± 1.44	0.801	0.828
	IC90	21.56 ± 0.84[b]	0.285	0.552
Cyclosporin	IC50	288.02 ± 14.43[b]	0.208	0.108
	IC90	559.05 ± 47.67[b]	0.640	0.169
Cytosar-U	IC50	0.0069 ± 0.0007	0.157	0.610
	IC90	0.014 ± 0.0014[b]	0.381	0.660
Cytoxan	IC50	40.37 ± 3.3	0.255	0.518
	IC90	79.34 ± 5.91[b]	0.518	0.296
Etoposide	IC50	0.97 ± 0.15	0.130	0.496
	IC90	1.64 ± 0.23	0.153	0.847
Fludarabine	IC50	0.30 ± 0.026	0.068	0.460
	IC90	0.58 ± 0.062	0.095	0.781
5-Fluororuacil	IC50	0.71 ± 0.10	0.114	0.178
	IC90	1.46 ± 0.17	0.082	0.103
Gemcitabine	IC50	0.005 ± 0.0001	0.018	0.741
	IC90	0.008 ± 0.0001[b]	0.014	0.441
Indocin	IC0	50	—	—
Lindane	IC0	50	—	—
Methotrexate	IC50	46.19 ± 0.69	0.017*	0.941
	IC90	126.6 ± 19.62	0.171	0.489
Myleran	IC50	2.78 ± 0.16	0.018*	0.767
	IC90	4.76 ± 0.16	0.009*	0.140
Retrovir	IC50	24.27 ± 4.84	0.653	0.955
	IC90	48.91 ± 10.11[b]	0.560	0.881
Taxol	IC50	0.004 ± 0.0002	0.023*	0.743
	IC90	0.007 ± 0.0001	0.008*	0.224
Teniposide	IC50	0.052 ± 0.022	0.58	0.847
	IC90	0.11 ± 0.031	0.487	0.887
Thioguanine	IC50	1.19 ± 0.09	0.448	0.422
	IC90	2.32 ± 0.024	0.011	0.145
Thorazine	IC50	6.23 ± 0.51	0.081	0.260
	IC90	10.02 ± 0.69	0.099*	0.242
Zovirax	IC50	61.24[b]		
	IC90	104.2[b]		

[a]The homogeneity of IC values obtained by laboratories in different experiments were analyzed by ANOVA.
[b]Values extrapolated on the regression curve out of the actual drug doses tested.
*If $p < 0.05$, we conclude that IC values interlaboratory or intralaboratory/interexperiments differ significantly.

Cytoxan, and by one laboratory for Retrovir. For six drugs (Indocin, Lindane, Methotrexate, Zovirax, Carmustine, and Cyclosporin), none of the laboratories was able to determine the IC90 value.

The analysis of the IC values determinable by each laboratory suggested that the extrapolated IC90 values for Carmustine, Cyclosporin, Cytosar-U, Gemcitabine, and Zovirax had a sufficient degree of reliability with the McIC > 50%. For the remaining drugs, the extrapolated IC90 values were imprecise because the McIC was lower than the 50%.

A very high intralaboratory homogeneity was observed (only the IC90 determination for Teniposide showed a $p < 0.05$). The interlaboratory variability was lower than that observed in the human model and concerned five drugs: IC50 (Fludarabine), IC90 (Etoposide), IC50 and IC90 (Cyclosporin, Myleran, and Teniposide). As in the human model, the interlaboratory variability in the murine study influenced the IC determination for 5 drugs out of 20 (25%) and 8 IC determinations out of 40 (20%).

In the human model, the interlaboratory variation observed

TABLE 3
IC50 and IC90 Values (Expressed as μg/ml) Determined in Murine Model

Drug	IC value	Mean ± SD	*P* values[a]	
			Interlaboratory	Intralaboratory/interexperiment
Adriamycin	IC50	0.0054 ± 0.0002	0.147	0.058
	IC90	0.0108 ± 0.0005	0.142	0.109
Bleomycin	IC50	2.05 ± 0.34	0.692	0.911
	IC90	5.30 ± 0.15	0.128	0.816
Carmustine	IC50	10.24 ± 1.11[b]	0.336	0.327
	IC90	17.29 ± 1.93[b]	0.350	0.335
Cyclosporin	IC50	91.11 ± 3.49	0.029*	0.297
	IC90	198.5 ± 5.52b	0.022*	0.430
Cytosar-U	IC50	0.11 ± 0.007	0.895	0.475
	IC90	0.17 ± 0.006	0.489	0.071
Cytoxan	IC50	164.25 ± 16.82[b]	0.566	0.559
	IC90	296.97 ± 51.94[b]	0.671	0.559
Etoposide	IC50	0.96 ± 0.127	0.431	0.422
	IC90	1.79 ± 0.023	0.007*	0.176
Fludarabine	IC50	11.08 ± 0.16	0.042*	0.0548
	IC90	17.17 ± 0.93	0.369	0.517
5-Fluororuacil	IC50	0.14 ± 0.015	0.248	0.513
	IC90	0.24 ± 0.018	0.085	0.295
Gemcitabine	IC50	0.0097 ± 0.0003	0.075	0.770
	IC90	0.016 ± 0.0007	0.230	0.889
Indocin	IC0	50	—	—
Lindane	IC0	50	—	—
Methotrexate	IC50	119.62 + 10.16[c]	—	—
	IC90	197.41 + 8.57[c]	—	—
Myleran	IC50	11.62 ± 0.57	0.0339**	0.352
	IC90	22.298 ± 0.78	0.0195*	0.615
Retrovir	IC50	50.5 ± 10.3b	0.749	0.338
	IC90	101.82 ± 20.98b	0.833	0.350
Taxol	IC50	0.0039 ± 0.0002	0.092	0.346
	IC90	0.0061 ± 0.0004	0.114	0.255
Teniposide	IC50	0.05 ± 0.002	0.012*	0.188
	IC90	0.102 ± 0.002	0.0076*	0.026*
Thioguanine	IC50	0.418 ± 0.019	0.305	0.167
	IC90	0.79 ± 0.040	0.14	0.555
Thorazine	IC50	6.16 ± 0.64	0.43	0.901
	IC90	9.22 ± 0.41	0.566	0.727
Zovirax	IC50	32.99 ± 3.62	0.783	0.699
	IC90	49.89±5.03[b]	0.587	0.787

[a]The homogeneity of IC values obtained by laboratories in different experiments were analyzed.
[b]Values extrapolated on the regression curve out of the actual drug doses tested.
[c]Determinable in only one laboratory.
*If $p < 0.05$, we conclude that IC values interlaboratory or intralaboratory/interexperiments differ significantly.

seems have been due to the use of different donors rather than to CBC variations in methodology. In fact, the very rare occurrence of intralaboratory variation during the human CFU-GM study, and the normalization of the colony counts to the D_0 control, suggest that biological variation in the CBC response across individual donors may contribute more. In the murine model, the interlaboratory variation observed seems more attributable to the technical methodology, because the bone marrow was obtained from syngenic mice.

Prediction of Human MTD

The IC90 values for the murine and human CFU-GM generated with our SOP were applied to the algorithm of the prediction model according to two different groups of drugs, as reported in Tables 4 and 5. T4 T5

Table 4 shows the human/murine IC90 ratios calculated for 10 drugs for which it was possible to determine IC90 values by interpolation of the regression curve within the range of the

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TABLE 4
Prediction of Human Maximum Tolerated Dose (MTD) for Drugs with Determinable IC90 Values

Drug	IC90 ratios (human/murine)	Actual[a] murine LD10 (mg/m^2/dose)	Predicted human MTD (mg/m^2/dose)	Actual human MTD (mg/m^2/dose)	Successful prediction? (yes/no)
Adriamycin	0.926	11.1[19]	10.2	22.5[1]	Yes
		13.5	12.5	5	Yes
Bleomycin	0.428	27.9[21]	11.9	15[2]	Yes
Etoposide	0.912	23.1[20]	21.1	54[21]	Yes
Fludarabine	0.034	1008.9[22]	34.3	25[3]	Yes
5-Flurouracil	5.98	66[21]	394	740[4]	Yes
		96	574	1295	Yes
Myleran	0.21	90[21]	18.9	24.2[5]	Yes
Taxol	1.19	69.6[28]	82.8	40[6]	Yes
Teniposide	1.6	15.9[30]	25.4	80[7]	Yes
Thioguanine	2.86	27.3[19]	78	35[8]	Yes[b]
		156	446	1000	Yes[c]
Thorazine	1.03	158[22]	162	79.3[9][a]	Yes

Note. Human dosages in mg/day were converted into mg/sqm assuming 70 kg body mass. References: (1) Creasey *et al.* (1976); Vogelzang *et al.* (1985); (2) Lokich and Curt (1985); Dorr and Von Hoff (1994); (3) Hutton *et al.* (1984); (4) Moertel *et al.* (1972); Seifert *et al.* (1975); (5) Galton and Till (1955); Petrakis *et al.* (1954); (19) Southern Research Institute (1980); Intramural Toxicology Branch (1984); (20) Nissen *et al.* (1972); (21) Southern Research Institute (1980); (22) Intramural Toxicology Branch (1984); (28) Intramural Toxicology Branch (1984); Schweikart (2000); (30) Stahelin (1970).

[a]Adjusted for 10% oral bioavailability[9].

[b]Based on qd × 5 regimen.

[c]Based on qd × 1 regimen.

TABLE 5
Prediction of Human Maximum Tolerated Dose (MTD) for Drugs with Extrapolated IC90 Values Lying beyond the Test Concentration Range

Drug	IC90 ratios (human/murine)	Actual[a] murine LD10 (mg/m^2/dose)	Predicted human MTD (mg/m2/dose)	Actual human MTD (mg/m2/dose)	Successful prediction? (yes/no)
Carmustine	1.48	93[23]	137.6	400[10]	Yes
Cyclosporin	2.82	180[24]	508	740[11]	Yes
Cytosar-U	0.081	504[19]	40.1	350[12]	No
		138[19]	11.2	130[12]	No
Cytoxan	0.27	1134[22]	306	350[13]	Yes
Gemcitabine	0.53	60[25]	31.8	210[14]	No
Indocin	N.D.	>27[26]	N.D.	107[15]	Yes[b]
Lindane	N.D.	N.D.[27]	N.D.	N.D.[16]	Yes[b]
Methotrexate	0.64	6.6[28]	4.2	1.8[1]	Yes
		28.35[28]	18.1	15[1]	Yes
Retrovir	0.47	810[29][a]	380	1300[17]	Yes
Zovirax	2.0	495[22]	990	5328[18]	No

Note. References: (23) Davis *et al.* (1988); (24) Bennett *et al.* (1985); (25) Veerman *et al.* (1994); (26) Robinson *et al.* (1967), Southern Research Institute (1980); (27) Starr and Clifford (1972); (28) Intramural Toxicology Branch (1984), Schweikart (2000); (29) Gallicchio and Hughes (1992), Hamburger *et al.* (1993), Manouilov *et al.* (1995); (19) Southern Research Institute (1980), Intramural Toxicology Branch (1984); (22) Intramural Toxicology Branch (1984); (10) Boardman and Hart (1967), Anonymous (1970), Yeh (1985); (16) Aks *et al.* (1995), Davies *et al.* (1983); (1) Creasey *et al.* (1976), Vogelzang *et al.* (1985); (17) Pizzo *et al.* (1988); (18) Fletcher *et al.* (1989).

[a]Adjusted for bioavailability after oral dosing.

[b]Using IC50 values in an alternative prediction model.

*Note on mouse LD10 values: Mouse LD10 values were obtained from NCI toxicology files and the literature. When multiple studies were presented, the most important criteria for selecting a study was identical dosage regimen, followed by the lowest LD10 value from identical studies (assumes poor drug solubility explains the higher doses), followed by BDF1 then CDF1 then other mouse strains. Mouse sex was not a criterion for selection; in cases with separate studies in the sexes of one strain, the values from the two studies were averaged.

actual drug doses tested. Human MTD values are accurately predicted for 100% of the tested drugs in this group.

Table 5 shows the IC90 ratios for the 10 drugs that required extrapolation of IC90 values from the regression curve outside the dose range tested. The prediction model applied to this group of drugs "accurately" predicted 7 drugs out of 10 (70%) but failed to accurately predict the human MTDs of Cytosar-U, Gemcitabine, and Zovirax.

DISCUSSION

General Considerations on the MTD for Neutropenia

The IC90 value is the concentration that inhibits CFU-GM colony formation by 90%, and half of the tested compounds were potent enough to inhibit both the human and murine CFU-GM to this degree within the range of concentrations that were completely soluble in water.

Severe toxicity in this context means life-threatening toxicity, in which supportive medical care may be required for recovery and in which the marrow MTD will equal the MTD for the human being as a whole. In cases where hematopoiesis is less sensitive to the toxicant than another organ system, the human MTD will be lower than the bone marrow MTD. However, it is important to note that the human MTD will never be higher than the bone marrow toxicity, at least as irreversible in the remaining life span of the individual. Whether a toxicant will cause neutropenia depends entirely on whether the other organ systems can tolerate this exposure level. Therefore, according to these above-mentioned concepts, our validated prediction model predicts the human dose that will be associated with life-threatenig neutropenia.

It is also important to note the fundamental departure of this validation study from the customary toxicology strategy of hazard classification and the importance of this departure to the success of the study. Hazard classification depends on finding some *in vitro* characteristic that is highly associated with the toxicity of interest and not with other outcomes. At the time this study was planned, it was widely appreciated that short-term exposure to many toxicants does not necessarily cause hematoxicity *in vivo*, despite the fact that inhibiting proliferation of the neutrophil precursor in the bone marrow leads to reversible neutropenia. This reasoning led to the somewhat obvious conclusion that the level of exposure relative to a toxicant's potency is the determining factor for the clinical outcome, rather than the potency or degree of *in vitro* inhibition *per se*.

Therefore, all of the compounds must be classified as potentially hematotoxic, and the only issue to be predicted by *in vitro* alternatives is the dose level that will affect the bone marrow.

Abandoning hazard classification led to the realization that the concept of negative and positive controls was not useful for the validation process. Instead, the study required a set of test compounds that reflected a range of potencies at the marrow targets and the widest possible range of interspecies differences in tolerated doses between mouse and human. "Negative control" in this context means a compound that does not cause hematotoxicity *in vivo* because its substantially greater potency on another organ system prevents exposure levels from becoming high enough to affect the bone marrow. This argument provided the rationale for the dependence of the validation study on the use of oncology drug products as test articles. This set of compounds has two additional advantages as test substances: There are complete data sets on their murine and human toxicology from product safety studies and controlled clinical trials, respectively, and it is ethical to obtain human dose–toxicity relationships, including life-threatening exposure levels, in the context of closely monitored clinical trials. Almost all of the oncology products tested in the current study can be administered to humans at exposure levels that are myelosuppressive, because they have narrow therapeutic indices that require toxicity to achieve efficacy, and for most of them neutropenia is the most common dose-limiting effect. The exceptions are Bleomycin, which causes dose-limiting pulmonary toxicity without myelosuppression, and probably Cytoxan, which is converted to the active 4-hydroxycyclophosphamide metabolite and other metabolites that may contribute to its toxicity. The toxicity profile of Cytoxan itself in the absence of metabolic activation is not known. Clinical trials have shown that antivirals such as Retrovir (zidovudine, "AZT") and Zovirax (aciclovir) also cause dose-limiting toxicity to hematopoiesis. However, they are usually used at dosages that do not cause neutropenia, because their therapeutic indices are higher than those of oncology products. The remaining test compounds reach myelosuppressive levels only in the context of overdosage (Thorazine), or they were the validation study's negative controls (Cyclosporin and Indomethacin), the exposure levels of which could never reach myelosuppressive levels, because of severe organ toxicities occurring outside the bone marrow. This group of test items met the need for a very wide range of potencies at the marrow target in humans, including compounds that have such a low potency that the marrow MTD will be substantially greater than the human MTD. They also represent a wide range of mechanisms of action, structural diversity, and biophysical properties.

Considerations on the Validated CFU-GM SOP

This prevalidation-validation effort incorporated several key technical aspects not usually found in the common method of CFU-GM testing that were probably critical to the success of this study.

First, several chemical modifications to the Stem Cell MethoCult culture medium were made to avoid substances that could interfere with the toxicity of the test articles. 2-Mercaptoethanol is usually found in this culture medium because it

improves colony number and colony size, but, being a mercaptan and nucleophilic, it could react with many of the chemically reactive toxicants that might need to be evaluated in the CFU-GM test. Likewise, transferrin is a key component in culture media that support cell proliferation, but the ferrous iron that it carries, in the presence of the high oxygen tension that exists in most CO_2 incubators, can react with xenobiotics to produce free oxygen radicals. Among the test substances in this study, the iron-catalyzed generation of free radicals has been implicated in the toxicity of Bleomycin and Adriamycin. The potential of 2-ME and the ferrous iron to react with toxicants and thereby lower their effective exposure levels in the culture medium was only of minor concern, because the human and murine culture systems would have contained the same levels of 2-ME and transferrin; therefore, the relative measure of potency across species required by the prediction model would not have been affected. However, the management team was concerned about the products of these chemical reactions modifiying a test article's toxicity or exhibiting biological activity themselves in the CFU-GM test system and then misinterpreted this artifactual activity as that of the test compound.

Second, the growth stimulant of colony formation was limited to recombinant GM-CSF. Cell-line conditioned medium and cytokine/growth factor cocktails were avoided, because they stimulate colony formation by additional neutrophil precursor cells, which are less mature than the CFU-GM. The role of these immature cells in hematotoxicity is not yet clear, but *in vivo* modeling in mice has shown that they are probably not targets for acute toxicant exposure that causes reversible neutropenia. It is not possible to know whether the assay could have been validated without making these changes.

Third, the mouse MTD values used for prediction modeling, and the human MTD values to which they were compared, were derived from studies with the exact same route of toxicant exposure (usually intravenous, but also including oral) and similar dosing regimens. Given the schedule dependency of many marrow toxicants, it was very important to match route and regimen across species, so the true interspecies difference measured by the CFU-GM test, which is a difference in potency at the same target organ, could be detected accurately. Comparing, for example, one-day dosing in the mouse versus five consecutive days in the human can hide the true interspecies variation in susceptibility underneath an overwhelming effect of scheduling on toxicity.

Consideration of Model Predictivity

This validation study, performed with a panel of 20 drugs, confirms that the SOP developed for assaying human and murine GM-CFU generates reproducible IC90 values that were applied to our model for predicting acute systemic doses that will cause severe, reversible neutropenia in treated patients (marrow MTD). Because the intralaboratory variability was lower than the interlaboratory one, the best homogeneity for IC determination is attained when human and murine CFU-GM assays are performed in the same laboratory. The biological differences due to different donor cord blood cells is likely to contribute to the greater interlaboratory variation in human than mouse CFU-GM data. However, this variation was acceptable and did not compromise the predictive accuracy of the model. For 10 drugs, IC90 values were found within the range of the actual drug doses tested (defined as the actual IC90). However, for the other 10 drugs, extrapolation on the regression curve out of the range of actual doses tested was required to derive IC90 values (defined as the extrapolated IC90). Our method correctly predicted the MTD for 10 drugs that had actual IC90 values.

Lindane and Indocin were selected as negative controls, and they indeed were nontoxic to CFU-GM, failing to reach even predicted IC 90 values because of a general lack of CFU-GM *in vitro*. However, relatively accurate IC50 values were available from the human CFU-GM assay; therefore we used a secondary prediction model that stated that the peak (maximum) plasma concentration of any toxicant that did not cause acute neutropenia in humans will lie below its IC50 value in the CFU-GM assay. Note that this model does not involve interspecies comparisons, and the IC50 value was chosen because this seemed to be the greatest amount of CFU-GM loss that is not associated with neutropenia in a small number of studies (Parchment *et al.,* 1994; Parent-Massin and Parchment, 1998). For Indocin, 10.8–15.7 (avg 13.5) mcg/ml plasma concentration of drug causes severe toxicity in just 7% of patients, and it is usually not bone marrow suppression. These plasma concentrations lie below the IC50 value of 264 mcg/ml in the human CFU-GM assay, so this toxicant's risk of neutropenia was correctly predicted by the model. Several h after acute Lindane exposure, serum concentrations >0.2 mcg/ml cause seizure, >0.5 mcg/ml cause myonecrosis, and >1.2 mcg/ml cause death (Aks *et al.*, 1995; Davies *et al.*, 1983; Starr and Clifford, 1972). However, these cases of accidental exposure were not associated with any reported bone marrow suppression, even though an extensive emergency room workup was performed in each case to characterize the extent of overdose. The lethal plasma concentration of 1.2 mcg/ml lies far below the IC50 value of 188 mcg/ml in the human CFU-GM assay, so this toxicant is counted as a success in the performance of the prediction modeling.

It is very important to recognize that the lack of *in vitro* CFU-GM toxicity provides evidence that the assay specifically detects myelosuppressive agents. Xenobiotics that do not usually cause hematopoietic toxicity failed to inhibit the neutrophil progenitor.

For those having only an extrapolated IC90, the method correctly predicted the MTD for seven drugs. Two of the incorrect predictions were within 6-fold of the correct MTD, instead of the 4-fold range required by the model. The final incorrect prediction for Cytosar-U was about 10-fold in error.

However, it is important to note that these incorrect predictions underestimated the human MTD, and therefore, from the perspective of product safety, these predictions would have overestimated human risk rather than underestimated it (a more serious mistake) (Collins *et al.*, 1990).

It remains to be explained why the method failed to predict the MTDs for Cytosar-U, Gemcitabine, and Zovirax. It seems realistic to suspect that this was due to IC90 "extrapolation" out of the range of the actual doses tested. Given the simplicity of the prediction model, underprediction is likely to originate from two sources. One explanation could be the irregular-shaped dose–response curves that deviate substantially from the linear extrapolations used in the analysis to estimate IC90 values. Rather, these relationships may curve downward to more potent IC90 values than predicted. Second, three drugs with erroneous predictions are pyrimidine analogues, and it is possible that differences in the levels of endogenous natural pyrimidines that antagonize drug toxicity have not been accurately modeled *in vitro*.

Three drugs that were tested in the prevalidation study were not included in the validation phase: Topotecan, PZA, and Flavopiridol. The method also correctly predicted the human MTD for these drugs during that study. Therefore, when considered in total, the method and the prediction model correctly predicted the human MTD for 20 drugs out of 23 (87%). Coupled with the reproducibility of the SOP application, this 87% predictivity and the 94% predictivity for nonnucleoside structures (15 drugs of 16) confirm that the SOP and the prediction model can be considered scientifically validated in this study.

This favorable outcome suggests promising areas for further research in developing validated hematotoxicology tests: (1) verification of the introduction of naturally occurring pyrimidines and purines into the culture medium at physiological levels; (2) application of the SOP in a microtest (96-well plates) for high throughput screening of compounds; and (3) extension of this SOP to the rat and the dog. The greatest reduction and refinement in rodent and nonrodent use in toxicology will occur in these species, which are used throughout the world for product safety testing. Canine CFU-GM does not grow well in response to either murine or human GM-CSF, so a source of this cytokine will be required before the validation of CFU-GM assays for the dog will be possible.

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