Alternative Testing Systems for Evaluating Noncarcinogenic, Hematologic Toxicity

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Hematopoietic tissues are the targets of numerous xenobiotics. Clinical hematotoxicity is either a decrease or an increase in peripheral blood cell counts in one or more cell lineages—a cytopenia or a cytosis, respectively—that carries a risk of an adverse clinical event. The purpose of in vitro hematotoxicology is the prediction of these adverse hematotoxic effects from the effects of the toxicants on human hematopoietic targets under controlled experimental conditions in the laboratory. Building on its important foundations in experimental hematotoxicology and the wealth of hematotoxicology data found in experimental oncology, this field of alternative toxicology has developed rapidly during the past decade. Although the colony-forming unit—granulocyte/monocyte neutrophil progenitor is most frequently evaluated, other defined progenitors and stem cells as well as cell types found in the marrow stroma can be evaluated in vitro. End points have been proposed for predicting toxicant exposure levels at the maximum tolerated dose and the no observable adverse effect level for the neutrophil lineage, and several clinical prediction models for neutropenia have developed to the point that they are ready for prospective validation and implementation in both preclinical species and humans. Known predictive end points are the key to successful comparisons across species or across chemical structures when in vitro dose-response curves are nonparallel. Analytical chemistry support is critical for accurate interpretation of in vitro data and for relating the in vitro pharmacodynamics to the in vivo pharmacokinetics. In contrast to acute neutropenia, anemia and acute thrombocytopenia, as well as adverse effects from chronic toxicant exposure, are much more difficult to predict from in vitro data. Pharmacologic principles critical for clinical predictions from in vitro data very likely will apply to toxicities to other proliferative tissues, such as mucositis. — Environ Health Perspect 106(Suppl 2):541–557 (1998).
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In Vitro Hematotoxicology as an Alternative Toxicology

Clinical toxicology can be defined as the study of the clinically significant perturbations, caused by xenobiotic and/or therapeutic exposure, which are adverse in nature (harmful) for the patient. This paper focuses on the prediction of clinically significant, adverse perturbations in peripheral blood cell counts. It addresses benign lesions in the hematopoietic system but does not consider leukemogenesis.

The field of hematotoxicology includes the study of adverse effects of toxicants on mature blood cells and also the precursor cells in the hematopoietic (blood forming) tissues. There are established techniques for assessing adverse effects of xenobiotics on mature blood cells. More recently, the availability of recombinant hematopoietic growth factors makes possible the evaluation of adverse effects against the blood-forming precursor cells as well. Thus, it is now possible to study human hematotoxicology in the preclinical setting. Because it is possible to study the effects of a toxicant on its actual target cell, it seems reasonable to expect in vitro hematotoxicology to be highly predictive.

Fundamentally, toxicology has two goals: identification of the tissues that are susceptible to the toxic effects of the xenobiologic and determination of the level of acute and chronic exposures (doses) that these tissues can tolerate without clinical consequences. The first goal is qualitative; it necessarily involves comparative toxicology in multiple tissues. This comparison is most efficiently completed in vivo, where all organs can be exposed simultaneously. A similar evaluation performed ex vivo necessarily requires the assay of multiple human tissues under identical conditions, which at the present time is not technically possible. The estimation of the acceptable level of human exposure from in vitro hematotoxicology data assumes that the toxicant’s most potent effects are toward the bone marrow, i.e., hematopoietic tissue is the most sensitive of the human tissues to toxicity. By its nature, hematotoxicology complements, refines, and actually improves standard toxicology testing (usually in vivo), which, in this case, is required to identify hematopoiesis as the most likely tissue target of the toxicant in humans. The second goal is quantitative and estimates the level of toxicant exposure that can be tolerated by the target tissue. This is critical for accurate risk assessment and establishment of reasonable regulatory limits on exposure. In this case, ex vivo evaluation must involve evaluation of toxicity to only the target tissue or cells derived from it and in vitro hematotoxicology meets this second goal.

The laboratory techniques for evaluating the effects of a toxicant on human hematopoietic tissue are relatively straightforward, and there is a wealth of data on the toxic effects of xenobiotics on hematopoietic target cells. Most laboratories have the
capability of performing all of the ex vivo tests described here. In contrast, the interpretation of the data and the quantitative prediction of the acceptable level of clinical exposure from the data are much more difficult. It is this latter arena in which we have specialized. We have investigated prediction models that translate the data into predicted hematologic perturbations as a function of toxicant exposure levels. 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. The former is often termed the no observable adverse effect level (NOAEL), whereas the latter is termed the maximum tolerated dose (MTD). Human exposure at the MTD is undesirable except for anticancer agents; with this exception, permissible exposure limits (PELS) are set for all regulated products from the NOAEL.

Substantial pharmacologic issues arise during the prediction of clinical outcomes from in vitro data. Ex vivo assays determine the concentration and exposure duration that cause toxicity. In contrast, the regulation of human exposure requires that decisions be based on units of dose or dose intensity (i.e., mg/m² or mg/kg per unit of time). The prediction of a dose that produces the in vitro concentration–time–effect relationship in vivo involves pharmacokinetics—the disposition of xenobiotic throughout the body. By analogy to animal and clinical toxicology, in vitro hematotoxicology aims to determine the no observable adverse effect concentration (NOAEC) and the maximum tolerated concentration (MTC) of toxicant under a specific schedule of exposure. The pharmacokineticist can then calculate the NOAEC and MTD doses that produce these NOAECs and MTCs. The NOAEC and the MTC are two end points useful in regulatory science sought during in vitro hematotoxicology studies.

The success to date lies primarily in the identification of the in vitro inhibition concentration value that is the MTC. An international validation study sponsored by the European Centre for the Validation of Alternative Methods (ECVAM) is underway to evaluate the predictive value of this putative MTC for clinical neutropenia (1). Results from this formal evaluation of in vitro hematotoxicology can be expected by January 1999. Because a wealth of detailed preclinical and clinical pharmacokinetic and hematotoxicity data exists for antineoplastic agents, these compounds serve as prototype hematotoxicants to elucidate the principles of predicting the NOAEC and the MTC. Once the prediction models are developed with antineoplastics, it is expected that they will generally be applicable to all other xenobiotics.

The Hematopoietic System as a Prototype for in Vivo and in Vitro Toxicology

Circulating Blood Cells and the in Vivo End Point

The blood of mammals contains a variety of differentiated cell types with specific functions. Red blood cells (erythrocytes) deliver oxygen; platelets contribute to clot formation; and lymphocytes, monocytes, and granulocytes provide resistance to infectious organisms and foreign materials. The granulocytes and platelets have a half-life in the circulation of only a few hours and a few days, respectively, whereas erythrocytes have a much longer half-life of several months. The proportion of white blood cells that are lymphocytes or granulocytes differs across mammalian species, and this must be kept in mind when interpreting toxicologic data. For example, rodents exhibit a lymphocyte:granulocyte ratio of about 4:1 whereas humans and dogs show a 1:4 ratio. A toxicant that causes severe neutropenia in the absence of lymphocytopenia will only be detected during rodent toxicology studies if differential counts are made on the leukocyte population. Otherwise, the maximum adverse effect will be a 20% reduction in white blood cell counts, which is likely to be considered unremarkable. Furthermore, it is important that validation studies of laboratory end points correlate the in vitro data to the correct in vivo end point. Assays to predict neutropenia need to be correlated with neutrophil counts, not with leukocyte counts.

Hematopoietic Cells as Producers of New Blood Cells

New blood cells must constantly be produced to maintain the peripheral blood cell counts, and under healthy physiologic conditions there is a balance between new cell production and cell loss that leads to the constancy of blood cell counts observed clinically. The hematopoietic tissues that produce new blood cells are primarily located in the bone marrow in humans but in both bone marrow and spleen in rodents and sometimes dogs. Hematopoietic tissue contains a continuum of increasingly differentiated elements within all blood cell lineages. Developing myeloid, erythroid, megakaryocytic, and lymphoid cells can be distinguished cytologically by the trained eye or by histochemistry for lineage-restricted features. The highly undifferentiated cells are progenitors of the immature blood cells. There are also hematopoietic stem cells in this undifferentiated population. Fixed stromal cells (both fibroblastic and histiocytic) and T lymphocytes in these tissues may exert some regulation over hematopoiesis.

The progenitors of the hematologic lineages will generate clonal colonies in vitro in semisolid media in response to specific cytokine combinations (2–4), hence the name colony-forming units (CFUs). The myeloid lineage contains the CFU-granulocyte/monocyte (CFU-GM) and the more mature progenitors, CFU-granulocyte and CFU-monocyte, which produce pure granulocyte or monocyte colonies, respectively (5–7). This lineage also contains progenitors for CFU-erythroid or CFU-basophil. The erythroid lineage contains the CFU-erythroid, which forms hemoglobinized colonies in response to erythropoietin (8–10), and the burst-forming unit-erythroid (BFU-E), which produces large, multilineage colonies of hemoglobinized cells in response to erythropoietin plus cytokines with burst-promoting activity (9,11–19). The megakaryocytic lineage contains the CFU-megakaryocyte (CFU-Mk) (also called CFU-Meg) (20–22), which responds to many cytokines, including the c-mpl ligand (23–30). By analogy to BFU-E, several laboratories report a BFU-megakaryocyte, which forms large, multifocal colonies (31,32). The lymphoid lineages also contain progenitors that form clonal colonies of B or T cells when stimulated with interleukin(IL)-7 and IL-2, respectively (33–35).

In addition to these lineage-restricted progenitors, hematopoietic tissue contains immature progenitors that form colonies containing multiple myeloid lineages (36–42). One such progenitor is CFU-granulocyte, erythroid, megakaryocyte, and monocyte (CFU-GEMM). As the name implies, colonies formed by this pluripotent progenitor are distinguished by the presence of cellular elements from all of these lineages. Several CFUs lie close to the hematopoietic stem cell developmentally. The high proliferative potential–colony-forming cell (HPP-CFC) produces a very large colony that contains primarily cells with a blastlike cytology, a small proportion of which will reform a clonal colony after
recloning (43–50). The long-term culture-initiating cell (LTC-IC) is a cell with multilineage potential found at low frequency in the marrow, probably positioned prior to the HPP-CFC in myelopoiesis (51–54). LTC-IC cells exhibit several characteristics of stem cells, including some capability of self-renewal, maintenance of both lymphopoiesis and myelopoiesis, and long-term reconstitution of a lethally irradiated host (55–64).

Bone Marrow Stroma

In contrast to the proliferation and expansion of tissue mass by hematopoietic cells, the primary function of the stroma is the nurture and support of developing blood cells (52,65–67). However, there is a stromal colony-forming unit called CFU-fibroblastoid (CFU-f), which produces a colony composed of adherent cells exhibiting morphologic features of fibroblasts, adipocytes, and other stromal elements (65,68). Although proliferating CFU-f is highly sensitive to many bone marrow toxicants in vitro, it is unclear whether the CFU-f is an in vivo target cell of toxicants that affect replicating cell types.

Interactive Cultures of Hematopoietic and Stromal Cells

Cultures of hematopoietic cells combined with stromal support cells can be maintained for several months in the absence of exogenously added cytokines by adhering to prescribed methodology (69–72). These so-called Dexter cultures sustain myelopoiesis at nearly steady-state levels for several weeks, after which time myeloid cell output begins to decline. It is possible to quantitate the output of progenitors or mature myeloid cells over time in these cultures. Likewise, long-term marrow cultures called Whitlock–Witte cultures sustain lymphopoiesis (73–76). Modifications of cell culture conditions can switch the cultures between lympho- and myelopoiesis, making it possible to evaluate both lympho- and myelopoiesis from the same cell culture (56,77). Because cytokines are not added to these cultures, they are thought to model closely the steady-state hematopoiesis that occurs in vivo. However, the progressive decline in mature cell output indicates the need to develop culture methodology for maintaining hematopoietic tissues in a homeostatic state.

Multidisciplinary Application of in Vitro Hematotoxicology

A great variety of toxicologic problems have been investigated using in vitro assays of hematopoiesis. The following examples have been selected to illustrate the wide-ranging usefulness of in vitro hematotoxicology. For more detailed discussions about study design and interpretation, see several recent reviews (1,78–93).

Toxicity to the Mature Blood Cell Compartment

Toxicity affecting primarily the mature, differentiated compartment usually manifests clinically as rapid-onset cytopenia. An example of this toxicity is phenylhydrazine-induced anemia caused by direct hemolysis of erythrocytes (10). However, hematologic toxicity following xenobiotic exposure can also manifest clinically as leukocytosis. Eosinophilia associated with exposure to IL-2, lithium, or a contaminant in over-the-counter preparations of L-tryptophan is due to toxicant-induced secretion of hematopoietic growth factors from mature blood cells (94–101). Xenobiotic-induced leukocytosis can also be due to abnormally high neutrophil or lymphocyte counts (102–105). CFU assays usually do not play a critical role in investigating this type of xenobiotic toxicity unless it cannot be explained by increased cytokine secretion.

Toxicity to the Progenitor Compartment

The mechanism of hematotoxicity most frequently and thoroughly studied in vitro is the acute effects of toxicant on marrow progenitors like CFU-GM (referred to as CFC-c in the older literature) and CFU-Mk. Toxicity is quantified from the number of surviving progenitors as a function of exposure level under maximally stimulatory cytokine concentrations.

Substances such as antineoplastics, biologic toxins, and ionizing radiation (106–116) destroy the rapidly dividing marrow progenitors, and a single exposure can result in an acute yet reversible neutropenia or thrombocytopenia 4 to 20 days later. A rapid repopulation of the progenitor compartment precedes recovery of peripheral counts by several days (106,117–124). The assumption of in vitro investigations of toxicity is accurate recapitulation of the in vivo toxicity in the in vitro model. In fact, in vivo toxicity to progenitors can be reproduced in vitro after direct exposure to pharmacologically relevant exposure levels of toxicants (78,81,83,84,106,125–136). Clinically achievable concentrations of myelosuppressive anti-human immunodeficiency virus nucleosides like azidothymidine (AZT) inhibit erythroid and myeloid progenitors (137–143) but the dideoxypurines, which do not cause anemia or neutropenia, do not (144,145). Clonogenic assays have also distinguished the relative contribution of each component of a multidrug regimen to therapy-related agranulocytosis (131,132,146,147).

Drug exposure levels that inhibit colony formation by approximately 50% do not cause neutropenia clinically (129,148). It is likely that the in vitro and in vivo data fail to correlate at these mildly toxic levels of exposure because the hyperplastic response of the marrow can compensate for this magnitude of progenitor loss. For example, there may not be any hematologic consequence from a 2-fold reduction in the frequency of CFU-GM if balanced by a 2-fold increase in marrow cellularity (no net gain in total CFU-GM). A direct correlation may exist between the decreases in clonogenic survival and absolute neutrophil counts only when toxicant levels cause such a severe lesion in the progenitor population that marrow hyperplasia cannot compensate.

In vitro assays can also be used to investigate cytopenias caused by noncytotoxic xenobiotics. For example, substances that reduce the number of differentiated blood cells produced per progenitor would be expected to decrease the size of the clonal colonies (the number of cells per colony), but not the number of colonies. Colonies that resemble CFU-GM colonies but are too small are named clusters. Thus, reduced CFU-GM colony formation accompanied by a shift in the distribution of colony size toward clusters argues against destruction of the progenitor. Trichothecene mycotoxins appear to shift the balance between proliferation and differentiation toward the latter (113–115). Interferons, the transforming growth factor beta (TGF-β) family, and tumor necrosis factors inhibit colony formation by CFU-GM, CFU-GEMM, and HPP-CFC (149–156). Experiments with TGF-βs indicate how subtle yet specific these effects on progenitors can be (149–151,154–156). The principles of predicting the parameters of neutropenia from in vitro data have not yet been established for this category of toxicants.

There is considerable confusion regarding a quantitative relationship between progenitor toxicity and acute anemia or thrombocytopenia. Megakaryocytopenia involves not only mitosis but also endoreplication, and it is unclear whether cell-cycle dependent toxicants are also endoreplication toxicants. For CFU-Mk assays, spontaneous colony formation occurs in vitro without
exogenously added cytokines; and colony formation is quantified based on a Δ value, i.e., the net increase in colony number due to cytokine stimulation. A cytokine-free control is included to quantify the number of colonies that form independently of added cytokine.

Toxicant-induced anemia is also difficult to predict from erythroid progenitor assays. Because erythrocytes circulate with a relatively short half-life, an acute toxicant-induced disruption of erythropoiesis is probably insufficient to cause anemia. For example, dipyrone inhibited not only CFU-GEMM and CFU-GM but also BFU-E in the presence of serum from a patient with drug-induced agranulocytosis and thrombocytopenia but not anemia (157). Also, ceftriaxime is an equally potent inhibitor of BFU-E and CFU-GM, although it is clinically associated with agranulocytosis (158).

The most difficult hematotoxicity to predict with in vitro assays is the progressive loss of one or more blood cell lineages during chronic exposure to a toxicant: agranulocytosis, pancytopenia, and aplastic anemia. In some cases the toxicity leaves a permanent dysfunction, while in other cases the toxicity resolves after identification and removal of the toxicant. The distinction between irreversible aplasias like aplastic anemia and the progressive yet usually reversible aplasias like agranulocytosis is important for proper application of in vitro assays. In practice, toxicity may not be definable as reversible or irreversible if the toxic insult cannot be identified or if the degree of permanent damage is insufficient to cause permanent symptoms. In the cases of irreversible marrow damage after multiple toxicant exposures, it is often impossible to know whether toxicity would have occurred after a single exposure.

Progressive yet reversible xenobiotic-induced cytopenia generally indicates a direct effect of toxicant or metabolite on hematopoietic cell types. CFU-GM levels in patients with drug-induced agranulocytosis are depressed relative to controls (91,159–164), and inhibition of myeloid and erythroid progenitors is a likely mechanism for beta-lactam–induced agranulocytosis (158) and contributes to phenylhydrazine-induced anemia (10). In these cases the in vitro progenitor assays correctly identified the mechanism of myelosuppression. Hypersensitivity of progenitors from susceptible individuals to the toxicant or a toxic metabolite can be demonstrated with progenitor assays (161,165–171). However, other xenobiotics (chlorpropamide, phenytoin, methimazole, valproate, disopyramide, and phenacetin) not only inhibit progenitors directly but also induce both cellular and soluble immune mechanisms, which inhibit hematopoiesis (161,163,172–176). Because immune constituents can contribute to hematotoxicity, it is important to eliminate effector cells when trying to prove a direct toxicity of xenobiotic to progenitors in vitro (168). Otherwise, the effects of inhibitory cytokines released by mature T cells and monocytes in response to toxicant may be measured instead, which would be a toxicity to the mature blood cell compartment rather than the progenitor compartment. Unfortunately, in many cases when the immune system contributes to hematotoxicity, in vitro studies using marrow from normal donors are not informative and in fact are usually inconclusive (167,170,177).

It is encouraging that several laboratories arrived independently at similar assay conditions for quantifying toxicity to hematopoietic progenitors (1,81–85,178,179). Micromass techniques for progenitors are also available when xenobiotic is in short supply (180).

**Toxicity to Bone Marrow Stroma**

The microenvironment is a target for toxicant injury (90), and assays of stromal function have been used to investigate the effects of toxicant exposure. Bone marrow stroma is more sensitive than hematopoietic progenitors to the toxicity of acute neutron irradiation but less sensitive to X rays, and hematopoietic stem cell and stromal cell repopulation, but not progenitor survival, are dependent on dose rate (109,112,124,181–184). Long-term bone marrow cultures based on Dexter’s method (69) have been used to investigate the agranulocytosis associated with ceftriaxime and other agents (82,158). In addition, the CFU-f is potentially a dose-limiting cell type for radiation and some quinones of benzo[a]pyrene (183–185). CFU-f is the most sensitive progenitor to AZT toxicity, and AZT causes perturbations in parameters of long-term bone marrow assays at clinically relevant concentrations (186). Both conventional and high-dose cancer chemotherapies cause permanent damage to the marrow microenvironment (111,148,187–192). However, clinical hematotoxicity parameters can recover and even normalize in spite of continuing in vitro measurements of impaired marrow function (148,182,189–195), casting doubt on the power of these in vitro assays to predict clinical outcome after chronic exposure.

**Conceptual Framework for in Vitro Hematotoxicology Studies**

Alternative hematotoxicology has benefited tremendously from the progress in experimental hematology over the past 40 years. Recombinant cytokines are available, and consequently a number of hematopoietic cell types can be assayed. In vitro hematotoxicology differs significantly from other traditional toxicities in that the actual target cell of the toxicant can be studied in the laboratory. In addition, human exposure–hematotoxicity relationships are available from clinical trials in oncology for quantitative correlation with in vitro data. Finally, there appears to be little disagreement over the standardized conditions to use for quantitative assays and several laboratories in this field have reached similar conclusions about experimental variables via independent studies (1,81,84,85,178,179).

**What Should Be Predicted?**

Clinical hematotoxicity resulting from xenobiotic exposure can be described by four parameters (Figure 1): severity of the change in blood cell counts (cytopenia or cytosis), time from exposure to the most severe toxicity, duration of the nadir, and time required for recovery from the toxicity (degree of reversibility). These parameters completely describe the clinical course and nature of xenobiotic-induced cytopenia and cytosis (88). These toxicologic parameters have several important characteristics. They are not linked to any assumptions about molecular mechanism; they are clinical end points measured in patients and therefore constitute the in vivo data that in vitro end points must predict, and they provide a quantifiable description of xenobiotic effects on human blood cell counts.

Because regulatory agencies must make decisions about human PELs even for compounds with unknown mechanisms of action, it is preferable to have mechanistically naive assays, i.e., assays predictive across broad mechanistic classes. Molecular mechanism is not trivialized by this approach; rather it is admitted that this information is not required to regulate the product or treat the intoxicated patient. For example, any compound that caused reversible thrombocytopenia of 10 days’ duration would be treated similarly, whether the xenobiotic disrupted
PREDICTING HEMATOLOGIC CONSEQUENCES OF XENOBIOTIC EXPOSURES

Figure 1. Four independent parameters describe acute hematologic toxicity in vivo. In leukocytosis, the parameters are modified to the maximum increase in leukocyte counts; time to the maximum, duration of the maximum, and time to recovery. In vitro hematoxicology can be focused on the prediction of these four parameters in the human prior to any actual human exposure. Initial efforts have focused on validation of in vitro systems for predicting the severity of the neutrophil nadir after acute (preferably single dose) exposure. ** indicates the time of acute toxicant exposure.

signal transduction or destroyed progenitors. We view mechanism-independent toxicology as generally useful and advantageous both because it prevents assay proliferation syndrome, in which a new assay must be created for each mechanistic class that must be regulated, and because it can contribute to the regulation of new products with poorly understood mechanisms of action.

The goal of in vitro hematoxicology is the prediction of these four parameters (in vivo end points) from end points obtained from assays of human hematopoietic function after in vitro exposure to the toxicant (84,88). Different in vitro end points, and perhaps even different hematopoietic assays, may be required to predict each in vivo parameter. Most studies focus on the prediction of the severity of the nadir.

Extending the Concept of Dose-Limiting Tissue to a Dose-Limiting Cell Type

Within any hematologic lineage there are a number of potential target cells for the toxicant: the mature cell compartment in the bloodstream, the progenitors in the marrow that make these blood cells, and the supporting cells in the hematopoietic tissues that regulate blood cell production. Most of these can be assessed for toxicity in vitro and it is important to determine which is the actual target cell of the toxicant in vivo. This is especially important because many toxicants may show some degree of in vitro toxicity to all of the cell types in the lineage but only the in vitro end point from the assay of the actual target cell population will be the most likely predictor of clinical toxicity. Given data from assays of mature blood cell lysis and dysfunction, progenitor survival and function, and stem cell and stromal cell function, how does one predict what will be the clinical manifestation of toxicant exposure?

It is a well-established principle of in vivo toxicology that the adverse effects of exposure are determined by the most sensitive of the exposed tissues. The most sensitive tissue is called the dose-limiting tissue, and in vitro toxicology must predict the ramifications of exposure in this tissue to predict clinical toxicity. By analogy, the manifestations of toxicant exposure in the dose-limiting tissue must be determined by its most sensitive cell compartment, which we have called the dose-limiting cell type (88). This analogy is helpful in the interpretation of in vitro data when a toxicant is evaluated on the entire spectrum of cell types that constitute a hematopoietic lineage. For example, the nature of the neutropenia caused by a toxicant will be determined in large part by whether mature neutrophils, immature neutrophils, myeloid progenitors, stem cells, or myelopoietic support cells are the in vivo target of the xenobiotic. Until more is understood from attempts at in vitro-in vivo correlations, it seems reasonable to assume that the dose-limiting cell compartment in vivo is the cell type most sensitive to the toxicant in vitro.

It is possible that by identifying the dose-limiting progenitor, we may predict the time to nadir. For the neutrophil lineage, this hypothesis postulates that neutropenia due to a lesion in an immature neutrophil progenitor population will take longer to appear than a lesion in a relative late-stage neutrophil progenitor pool. This has yet to be proven, however.

In Vitro Pharmacology

The in vitro assays are relatively simple to perform and a wealth of data can be generated in a short period. In general, the results of the test will be expressed as the change in an in vitro end point (Y axis) as a function of the level of toxicant exposure (X axis, usually in concentration units) using a clinically relevant duration of exposure (Figure 2A). The next step is to translate this in vitro data into clinical language and predict the clinical consequences of toxicant exposure. However, the way this goal has been phrased implies a subtle distinction from the conventional purpose of toxicology studies. Traditionally, the question is one of classification: Is this compound a hematotoxicant? However, it seems reasonable to assume that any toxicant will be a hematotoxicant if the other tissues of the body can tolerate high enough exposure levels. Certainly there will be compounds that inhibit CFU-GM in vitro but do not cause neutropenia, because exposure levels never reach a high enough level: for example, the nonmyelosuppressive nucleoside analog dideoxycytidine is in fact toxic to CFU-Mk and CFU-GEMM in vitro (116,144,145). Because toxicologic experience lends credibility to this assumption, the practice of classifying chemicals as hematotoxicants should be abandoned.

If classification is to be abandoned, what prediction should replace it? We have attempted to answer this question during the past 7 years. The PELs for most regulated products are based on the NOAEL dose, whereas antineoplastics are regulated based on the MTD. We have come to understand that the most useful in vivo end points are those that predict the level of systemic exposure to toxicants at these two doses of regulatory significance. Hence, the purpose of in vitro hematotoxicology is to predict the toxicant exposure levels associated with the MTD and the NOAEL doses. We have termed the predictive in vitro end points for these two exposure levels the
MTC and NOAEC, respectively. This is analogous to the determination of the no effect and maximum tolerated doses in \textit{vivo}, except that \textit{in vitro} systems use concentration units instead.

For many hematotoxic compounds the severity of toxicity is related to a pharmacologic measure of exposure called the area under the plasma concentration versus time curve (AUC). Thus, the hypothesis of \textit{in vitro} hematotoxicology is that the \textit{in vitro} AUCs at the MTC and the NOAEC are identical to the plasma AUCs at the MTD and the NOAEL, respectively. This strategy shifts the emphasis of the \textit{in vitro} study to end points that lie on the X axis, not the Y axis. We should not try to predict whether compounds inhibit neutrophil production: rather we should assume that they could, and determine the AUC at the NOAEL and MTD for the neutrophil lineage (Figure 2B).

This understanding clarified how to determine if the CFU-GM assay could be validated for predicting neutropenia and used in the regulatory setting (88). We concentrated exclusively on this hematotoxicity because the relationship between progenitor numbers and peripheral blood cell counts is simpler for neutropenia than for anemia or thrombocytopenia. Furthermore, to identify the principles and concepts to include in clinical prediction models, we restricted our initial studies to the simplest of toxicity, the reversible neutropenia caused by an acute toxicant exposure. This rationale also focused the ECVM validation study in hematotoxicology on the prediction of neutropenia (1).

### Predicting Toxicant Exposure (Plasma AUC) at the MTD from \textit{in Vitro} Data

Because of our interest in the clinical development of antineoplastic agents and its regulation, most of our \textit{in vitro–in vivo} correlation studies have focused on the identification of the \textit{in vitro} end point that is the MTC (Table 1). A quantitative study of pyrazoloacridine found close correlation between inhibition of \textit{in vitro} colony formation and the grade of neutropenia \textit{in vivo} (129). In this study, the AUC that caused grade 3 to 4 neutropenia in humans inhibited \textit{in vitro} colony formation of human CFU-GM by 90%. The result suggested that the 90% inhibition concentration (IC\textsubscript{90}) from the human CFU-GM assay was the MTC when this progenitor is dose limiting. This conclusion was confirmed and extended in the mouse by showing that the AUC at the IC\textsubscript{90} from the murine CFU-GM assay was associated with a 90% reduction not only in absolute neutrophil count but also narrow CFU-GM (R Parchment, unpublished data).

Subsequently, the IC\textsubscript{90} was the \textit{in vitro} end point that predicted the differential between human and mouse MTD for the topoisomerase I inhibitor topotecan, whereas other IC end points failed to predict the MTD differential from the nonparallel human and mouse concentration–toxicity curves. Preliminary results show that the IC\textsubscript{90} ratio correlates to the MTD ratio for seven of seven tested drugs. Current studies are examining whether the IC\textsubscript{90} is the MTC for other progenitor compartments and for antineoplastic agents with other mechanisms of action. It is possible that a 90% reduction in content might not be tolerated in other progenitor compartments, while confirmation of this putative MTC for CFU-GM and additional toxicants will help clarify prediction models and gain regulatory acceptance.

### Predicting Toxicant Exposure (AUC) at the NOAEL from \textit{in Vitro} Data

Although the NOAEL is used much more frequently than the MTD as the basis for setting PELs for regulated products, there is a lack of understanding about the maximum degree of loss of progenitors that does not result in clinical neutropenia. This issue directly relates to our understanding of the resilience of hematopoietic tissue to toxic insult and the compensatory mechanisms, such as marrow hyperplasia, that can overcome mild reductions in progenitor survival. For example, a 50% reduction in CFU-GM survival coupled to a 2-fold increase in marrow cellularity would not result in any net loss of neutrophil progenitors. During our \textit{in vitro–in vivo} correlation study in the Phase I trial of 9-methoxypyrazoloacridine (129), the greatest plasma AUC that did not cause neutropenia inhibited human CFU-GM by 35%. Although circumstantial, these results suggest that the IC\textsubscript{35} may be the NOAEC, i.e., the end point from which the AUC at NOAEL can be predicted. Unfortunately, the minimum number of progenitors required to maintain normal peripheral blood cell counts \textit{in vivo} has not yet been determined for any myeloid lineage in any species. Until the IC\textsubscript{35} is established as the \textit{in vitro} NOAEC, the IC\textsubscript{0} or perhaps IC\textsubscript{05} should be used to estimate the NOAEL \textit{in vivo} (1). Obviously, there is a pressing need to determine the exact relationship between progenitor numbers and peripheral blood cell counts in each of the myeloid lineages and then identify the most predictive IC value to use for each level of myelosuppression \textit{in vivo}. We are currently examining other compounds to determine if the IC\textsubscript{35} consistently predicts the AUC at the NOAEL.

### The Critical Importance of Identifying the MTC and the NOAEC

Knowing the NOAEC and the MTC simplifies what otherwise would be very
Table 1. Identification of the IC₉₀ as the MTC in CFU-GM assays.

| Toxicant                        | Species         | Evidence                                                                 | Reference                           |
|---------------------------------|-----------------|--------------------------------------------------------------------------|-------------------------------------|
| 9-Methoxypyrrozoloacidine       | Human Mouse     | In vitro–in vivo correlation with ANC Intentional exposure at IAUC₉₀ produced 85% reduction in ANC but not for IC₉₀ | Parchment et al. (129)              |
| Topotecan                       | Human and mouse | Nine-fold differential for both MTD and IC₉₀ but not for IC₉₀             | Erickson-Miller et al. (219)        |
| Flavopiridol                    | Human and rat   | Rat more sensitive than human at IC₉₀ but more resistant at IC₉₀ and rat MTD lower than human MTD | J Tomaszewski (unpublished data)    |
| Fludarabine, fazarabine, camptothecins, amonafide | Multiple species | IC₉₀ ratios and MTD ratios correlate                                      | J Tomaszewski (unpublished data)    |

Abbreviations: ANC, absolute neutrophil count; IAUC₉₀, AUC at the IC₉₀. The toxicants are antineoplastic agents that cause myelosuppression; these are useful test compounds because neutropenia is an acceptable risk of human exposure.

complicated data interpretation (87,88). For example, it is common practice to compare a new compound with a reference compound of known hematotoxicity in vitro. Suppose one obtains data from such a study that shows a crossing of the concentration–response curves and it cannot be explained by differential chemical stability (Figure 3). From these data, can one predict whether humans can tolerate higher, the same, or lower levels of the investigational compound than the reference compound? In other words, should the PEL be higher or lower than the reference compound? If one knows that the IC₉₀ is the MTC and that grade 3 to 4 neutropenia is an acceptable risk of human exposure (e.g., a cancer drug), then clearly the tolerated AUC is higher for the investigational product than for the reference compound. Assuming equivalent pharmacokinetics, the PEL can then be set higher for the new product than for the reference product. In contrast, if the IC₃₅ is the NOAEC and neutropenia is not an acceptable consequence of exposure to this product, the opposite conclusion would be reached. The PEL for the investigational product should be set lower than the reference product because its IC₃₅ is lower. This theoretical example illustrates how the interpretation of the in vitro data depends on the intended use of the product. For one use the PEL can be higher, whereas for another use it must be lower. This example also suggests how to choose a least hematotoxic analog from an in vitro assay when the analogs do not exhibit parallel curves. The least toxic analog is that with the least inhibition in an appropriate in vitro assay at the end point most relevant to the product’s intended use. Such data would be impossible to interpret without these X-axis concepts.

The identical issue arises when using in vitro hematotoxicology to directly compare human toxicant sensitivity to that of the preclinical animal species in which the product is tested in vivo. In this case, the question is whether the preclinical toxicology species under- or overestimates human PELs for myelosuppressive compounds. If the direct comparison results in parallel curves (Figure 4A) then this question is easily answered. The human:animal ratio at any IC value will provide an estimate of the difference in tolerance of the compound; and, because the IC₅₀ is the most accurately determined point on these curves, the IC₅₀ ratios should be used. However, consider the more typical case of nonparallel curves across species (Figure 4B). Without knowing which IC values to compare, it would be difficult to interpret these data and predict human tolerance. However, a comparison of the IC₉₀ end point across species shows that humans can tolerate more than dogs but less than rodents. Assuming there are no pharmacokinetic differences across species, the MTD differential will be proportional to the IC₉₀ differential. In contrast, if neutropenia is an unacceptable risk of exposure, the NOAEC will be used for comparison. Assuming the IC₃₅ is the NOAEC, one would predict that the human PEL could be set higher than the rodent PEL by an amount proportional to the in vitro differential in the NOAECs.

Clinical Prediction Models for Prospective Evaluation and Validation Trials

Several prediction models have been developed that use the MTC and the NOAEC as predictive in vitro end points for the plasma AUC at the MTD and the NOAEL, respectively. From this estimate and knowledge of human pharmacokinetics.

![Figure 3. Differences between the test article and the reference compound depend on the level of inhibition at which the comparison is made. Knowing which in vitro end points are the NOAEC and the MTC makes it possible to interpret such in vitro hematotoxicology results. Note that the NOAEC is lower for the new product than the reference toxicant; in contrast, its MTC is higher. This interpretation assumes neutropenia is the dose limiting toxicity for both compounds.](image-url)
and plasma protein drug binding, the doses that achieve these AUCs in the plasma of exposed humans can be determined. However, the models can only be applied to compounds that are expected to show dose-limiting neutropenia. This is a critical assumption for \textit{in vitro} hematoxicology, and \textit{in vivo} toxicology studies must indicate that neutrophil precursors are the dose-limiting target. If the dose-limiting toxicity of the investigational product was e.g., cardiotoxicity, determination of AUC at the MTC for CFU-GM would overestimate human tolerance and jeopardize patient safety. Some studies have used the IC$_{70}$ instead of the IC$_{90}$ as the MTC, and these two end points will be compared during some of the ongoing studies. The IC$_{70}$ may be more appropriate when estimating PELs for products that will be used in patients with impaired marrow function. Prospective evaluation of these models will be critical in gaining acceptance, and Phase I clinical trials (dose-escalation trials) of antineoplastic agents provide an excellent opportunity for such an evaluation. The following models focus on the prediction of MTD; prediction of the NOAEL would simply substitute the IC$_{35}$ or other NOAEC end point for the IC$_{90}$.

Prediction Model 1 is the simplest model. It can be used if human pharmacokinetic parameters are unknown or cannot be determined. Model 1 has the greatest level of uncertainty because it incorporates only pharmacodynamics. It is based on the idea that neutrophil progenitors can serve as a sentinel tissue for interspecies comparisons. Large interspecies differences in toxicant disposition (AUC as a function of dose) could lead to significant errors in the predicted MTD. Step 1: determine the MTD in an animal model; step 2: Determine the toxicity differential between the animal and human dose-limiting progenitor based on IC$_{90}$ values; step 3: adjust the animal MTD for the IC$_{90}$ differential; and step 4: adjust the MTD again for differences in free drug concentration between the animal and human (e.g., protein binding).

Prediction Model 2 can be used when the AUC cannot be measured at the IC$_{90}$ for the human progenitor but human pharmacokinetic parameters are known or can be predicted. This model assumes the marrow toxicant causes an AUC-dependent cytoxicity. The model adjusts the plasma AUC at the MTD in the animal studies for species differences in drug tolerance and plasma protein drug binding. Step 1: determine the plasma AUC at the MTD in an animal model; step 2: determine the toxicity differential between the animal and human dose-limiting progenitor based on IC$_{90}$ values; step 3: adjust the animal-derived plasma AUC by the IC$_{90}$ differential; step 4: adjust the plasma AUC for differences in free drug concentration between species; and step 5: using human pharmacokinetic parameters or estimated parameters, calculate the dose that gives the predicted AUC.

Prediction Model 3 aims to predict the actual human MTD for the neutrophil lineage. It should be the most accurate prediction model because it incorporates both human pharmacodynamics and pharmacokinetics. Step 1: identify the most sensitive neutrophil progenitor in human bone marrow; step 2: determine the IC$_{90}$ for the dose-limiting human progenitor; step 3: derive the \textit{in vitro} AUC at the IC$_{90}$ by integrating the $C(t)$ curve; step 4: translate the \textit{in vitro} AUC into \textit{in vivo} terms by adjusting for differences in free concentration under the two conditions of exposure (e.g., protein binding);
and step 5: using human pharmacokinetic parameters or estimated parameters, calculate the dose that gives the predicted AUC.

Summary
Although our knowledge of how to use in vitro hematotoxicity data is in its infancy, these examples illustrate the progress that has been made by assessing exactly what end point is needed, i.e., what clinical end point should be predicted. The realization that X-axis rather than Y-axis end points are required for prediction was in a sense a breakthrough that has made it possible to propose clinical prediction models for prospective evaluation and validation (1,88,129). A second important breakthrough was our realization that predicting neutropenia actually involves the prediction of four independent parameters that, when taken together, describe clinical neutropenia: severity at nadir, time to nadir, duration of nadir, and time to recovery (84,88). Progress to date has been exclusively in the prediction of the severity of neutropenia (88,129). We are not aware that any progress that has been made in predicting the other parameters from in vitro data. Therefore, assays for CFU-GM and other granulocyte progenitors can be considered useful for investigating the cellular mechanism underlying the severity of reversible neutropenia and for determining the level of toxicant exposure that will be associated with grade 3 to 4 neutropenia. The investigation of the other parameters of neutropenia with in vitro assays should be considered exploratory research rather than established testing methodology.

Detailed Planning of in Vitro Hematotoxicity Studies
This section briefly covers some of the details that must be addressed when planning an in vitro hematotoxicity study (84,87,88).

Analytical Chemistry
There is substantial variability in the stability of different products under the conditions of the in vitro bone marrow assay, and subtle changes in the culture conditions can dramatically alter in vitro bioavailability. For example, many campstheothins show large differences in stability as a function of the species from which the culture medium serum or albumin is derived (196–200). These data show that one cannot assume that chemically related compounds have identical protein binding or stability in vitro and in vivo (84,88). In addition, some toxicants bind extensively to the cell culture containers (201). Therefore, the absolute CXT curve of a test substance at the MTC and the NOAEC under the conditions of the bone marrow assay must be determined chemically to determine the AUC. Chemical analysis also provides dose confirmation. Highly misleading results can be obtained when comparing two compounds, one of which is chemically stable and the other unstable, or one that is highly soluble versus one that is partially soluble. Unstable compounds and poorly soluble compounds will appear less toxic than equally potent stable or soluble compounds simply because the former have lower in vitro bioavailability than the latter. Concluding from in vitro data that a compound is relatively nontoxic, when in fact the data reflect an artificial rapid decomposition of compound in the culture medium, could cause serious underestimation of clinical hematotoxicity. Thus, colony inhibition data must be accompanied by sound analytical data before one can make clinical predictions, and analyte quantitation is an important aspect of the ECVAM validation study of the CFU-GM assay (1).

Duration of Exposure
In vitro data should be obtained not only for relevant concentrations but also for relevant durations of exposure. The duration of in vitro exposure to each test substance should mimic as closely as possible the duration of in vivo exposure. For example, if in vivo exposure is multiday, brief in vitro exposures are unnecessary. Likewise, if the substance rapidly decomposes in vitro then only brief in vitro exposures should be used. In fact, prolonged in vitro exposures to unstable compounds expose the cells to potentially myelotoxic breakdown products that may not be present in vivo. The effects of decomposition on perceived toxicity become magnified as the time of compound exposure becomes much larger than the time of decomposition.

When toxicant exposure is brief, it must be decided whether to preexpose the hematopoietic cells to cytokine, stimulating their entry into the cell cycle, or to expose the hematopoietic cells immediately after isolation from the human tissue source. Toxicants that show cell cycle-dependent toxicity would be expected to show significant differences in toxicity between the two conditions. This is an important question of experimental design that needs exploration.

Endogenous (Naturally Occurring) Antagonists and Protein Binding of Toxicants
For maximized correlation between in vitro and in vivo data, the in vitro assays must take into account physiologic levels of endogenous biochemicals that influence the toxicity of the test compound. Obvious examples of endogenous antagonists include pyrimidines and purines and their nucleosides for toxicants that target nucleoside and nucleic acid metabolism, and glutathione and other nucleophiles for alkylating agents. There are also cotoxicants such as molecular oxygen for free-radical generating compounds. The concentrations of both antagonist and cotoxicants must be as close as possible to in vivo levels.

The plasma proteins that bind xenobiotics (albumin, α1-acid glycoprotein) are another class of substances endogenous to both the in vitro and in vivo testing environments that introduce another source of error during interpretation of in vitro data. One cannot assume that a particular compound will behave identically in plasma from different species (such as from humans in vivo but from bovine or equine sources for in vitro assays), and the problem is especially significant for compounds that circulate tightly bound to carrier proteins. The levels of these proteins in the culture medium can dramatically affect the apparent potency of the test substance via changes in free (unbound) concentration of the actual toxicant (84,88,196–203). However, it is not necessary to add specific plasma proteins to the culture medium for in vitro hematotoxicity assays because results can be corrected for plasma protein binding measured in other simpler in vitro systems (1). It is best to compare several toxicants under identical culture conditions to learn about intrinsic differences in end-organ sensitivity and then correct the in vitro data for differences in protein binding determined in other assays. To facilitate in vivo extrapolation of in vitro data, the concentration–inhibition curve from bone marrow assays should express percent of inhibition as a function of free concentration of xenobiotic in the medium rather than total concentration. From these curves plus measurements of free versus total concentrations in human or animal plasma, it is possible to make in vivo predictions from in vitro data.

Protoxicants and Metabolic Activation
Although many toxicants are inactivated by metabolism, some compounds are
metabolized to toxic species. Human bone marrow stroma contains cytochrome P450s (CYP450s) that can bioactivate several toxicants, including polycyclic aromatic hydrocarbons, benzene, and cyclophosphamide (92,204–210). However, the metabolic capacity of the marrow stroma is small compared to liver, and it cannot produce sufficient levels of metabolite to reliably detect toxicity in the in vitro assays (1,92). Several in vitro systems are available for producing human CYP450 metabolites: liver microsomes, liver slices, hepatocytes, and transgenic cell lines expressing particular CYP450 isozymes. Hepatocytes or transgenic cell lines expressing specific CYP450s can be cocultured in the in vitro assays as a physically separate cell layer (92). Alternatively, stable metabolites can be isolated and evaluated individually in the in vitro assays.

The ECVM validation study addresses several issues related to the selection of metabolizing systems and the in vitro study of prototoxicants (1). S-9 fractions, dog liver microsomes, and rat liver microsomes are commonly used to produce metabolites. However, analysis of putative hematotoxic metabolites should use species-specific sources of metabolizing enzymes, i.e., human microsomes on human bone marrow or rat microsomes on rat marrow, because of the potential for interspecies differences in bioactivation (e.g., iododoxorubicin [211]). Xenogeneic combinations are sometimes warranted to prove the species-specific nature of certain toxicity problems or the lack of relevance of animal toxicology findings to humans. In addition, to exclude any contribution of metabolites to hematotoxicity, they should be tested by coexposure with the parent compound to identify any synergism or antagonism that might affect hematotoxicity.

Sources of Human Hematopoietic Cells

Mononuclear cells and subpopulations like CD34+ cells can be isolated from various sources: remnant marrow from femur or rib after surgeries, iliac crest aspirates from volunteers, umbilical cord blood, and peripheral blood leukocyte preparations (31,32,39,48,53,64,70,117,150,155,178,179,212–216). Bone marrow-derived progenitors might be the most applicable to human toxicology, but cord blood-derived cells are more readily available for research in many countries. Pharmacologic differences, if they exist, between hematopoietic cells in adults versus cord blood may be most pronounced after exposure to cell cycle-dependent toxicants because these populations of cells show substantial differences in cell cycle status after isolation (217). One study that directly compared the chemosensitivity of human marrow and cord blood CFU-GM did not find any differences (218). In contrast, there are differences between marrow and cord blood progenitors and between mobilized and stationary progenitors in terms of autocrine cytokine stimulation, cell cycle status, and susceptibility to cytomegalovirus toxicity (150,217). Differences in cell cycle status between cord blood and bone marrow hematopoietic cells accentuate the need to determine whether exposure to cytokines to stimulate entry into the cell cycle affects the predictive accuracy of in vitro models that use short duration of exposure.

Thoughts on the Immediate Future of in Vitro Hematotoxicology

Staying Current with Experimental Hematology

Experimental hematology is identifying hematopoietic cell types faster than toxicologists can determine if they are targets of toxicants. It cannot be assumed a priori that any progenitor is a target of hematotoxicants, but one cannot afford to assume that newly identified cell types are not targets and then be wrong. An efficient strategy to determine which of these cell types are targets is simply to determine which ones show altered levels in toxicant-exposed animals or patients with a time course that is consistent with a direct toxic effect. Furthermore, a new progenitor population in a preclinical species that is a bona fide toxicant target but is not found in human marrow would create a serious toxicologic problem.

If mechanism-naive assays are advantageous in regulatory science, it is important to work toward the replacement of clonogenic assays with new assays that measure the rate of production of mature blood cells by hematopoietic tissues rather than by survival of progenitors. Kinetic measurements of cell output by toxicant-exposed progenitors would offer some advantages over clonogenic assays. First, the capability of the marrow to compensate for the loss of up to 30 to 40% of its progenitors via a hyperplastic response would be detectable. Second, initial rates of mature cell output by the exposed progenitor population could be analyzed sooner than colony formation (3–4 days vs 14 days), so efficiency and through-put would be increased. Third, assays that could quantify toxicant effects on the kinetics of mature cell production have the capability of detecting myelosuppression regardless of whether it is caused by cytotoxicity. Such an effect would be hard to detect and quantify using clonogenic assays (smaller colonies but no reduction in their number).

Nuances in Data Interpretation

Colony-forming assays contain additional information to colony size. One of these was discussed above: the toxicant that causes smaller colonies but does not decrease their number. Consider this question: If the result of compound exposure is to inhibit the development of the erythroid component in CFU-GEMM colonies, should this be scored as inhibition of CFU-GEMM (which it probably is not) or as inhibition of an early erythroid progenitor generated by the CFU-GEMM? If the latter, would percent inhibition of BFU-E give the same estimate of myelotoxicity as the percent reduction in the CFU-GEMM that contains erythroid elements? In the design of studies to evaluate these toxicants, it may be important to limit the duration of exposure to only a few hours so that lineage-restricted progeny generated by surviving CFU-GEMM are not exposed to toxicant. Prolonged exposures would confuse toxicity to CFU-GEMM with toxicity to the progeny of CFU-GEMM.

Predicting the NOAEL raises the issue of the definition of NOAEL. Should the NOAEL be defined only by a lack of clinical symptoms and pathologic changes or should it indicate a lack of cellular and molecular changes as well? Defining the NOAEL with ever more sensitive end points, from tissue to cell to molecular levels, does not account for the compensatory mechanisms that may provide for continued health in spite of constant exposure to xenobiotics. Should PELs for a xenobiotic be based on altered gene expression in the absence of any adverse clinical effects? Rather, should it be concluded that the indicator gene, although a marker of exposure, must have some threshold yet to be exceeded and that the change does not indicate risk? The definition of NOAEL is important in regulatory science, especially as technology becomes more sensitive at detecting minute changes and more facile at expedited quests for effects. These issues must be integrated into validation studies of the NOAEC for predicting the human NOAEL.
Recommendations for Research Support

Federal agencies can take the lead in accomplishing the following goals that will promote the validation, clinical acceptance, and regulatory usefulness of in vitro hematotoxicology:

- After the ECVAM study (1) validates the human CFU-GM assay for predicting the toxicant exposure level that causes grade 3 to 4 neutropenia, examine assays for the differentiated spectrum of myeloid progenitors in the marrow for predicting time to neutrophil nadir.
- Fund an evaluation of possible in vitro assays and end points to predict the duration of the neutrophil nadir and/or the time to neutrophil recovery. Currently, it is much easier to investigate marrow toxicity than marrow recovery. For example, there are no in vitro assays for predicting the clinical efficacy of cytokines used to stimulate hematopoietic recovery. Research is needed to know how to predict time to recovery, which is likely related to predicting irreversible hematotoxicity as well.
- Coordinate regulatory- and industry-based surveys of human bone marrow and cord blood uses and needs for in vitro hematotoxicology and propose solutions to obstacles that currently limit the use of human bone marrow in these assays, which is the actual human target tissue.
- Identify the NOAEC end point in the CFU-GM assay and prove that CFU-GM but not neutrophil counts decrease by this amount in vivo at this exposure level.
- Fund veterinary and clinical research to identify what progenitor stage(s) in the platelet and red blood cell lineages fluctuates in concert with peripheral blood platelet and erythrocyte levels.
- Fund an evaluation of all newly identified hematopoietic cell types defined by in vitro assays and determine if acute in vivo exposure to hematotoxic xenobiotics causes a decrease in their bone marrow levels, i.e., whether these newly identified cell populations are frequent targets for xenobiotics or not.
- Fund research into the replacement of clonogenic assays with new in vitro assays that quantify the rate of production of mature blood cells by toxicant-exposed hematopoietic cells.

Significance of in Vitro Hematotoxicology for Other Alternative Toxicologies

There are substantial ethical, political, financial, and regulatory pressures to replace and refine animal toxicology with alternative assays. Studies of the toxicant on its actual human target tissues would seem to be the best alternative. In vitro hematotoxicology does this, and as a result shows much promise for predicting clinical hematotoxicity, especially neutropenia caused by acute exposures. It provides an opportunity to obtain human toxicology and pharmacology information in the laboratory setting and an experimental basis for selecting an animal model for investigating clinical hematotoxicity.

The in vitro assays of xenobiotic effects on human hematopoiesis can be viewed as prototypes of future in vitro toxicology assays that will reveal concepts and principles of clinical prediction. The hierarchical structure of stem cells and progenitors in the hematopoietic system likely reflects similar hierarchies in other renewing tissues of the body, such as the gastrointestinal mucosa. The emerging principles for prediction described in this paper will be applicable to toxicity in these other renewing tissues once clonogenic assays for epithelial progenitors are developed and the colony-stimulating assays for these other renewing tissues as well. Other end points and principles of clinical prediction will be needed for these more troublesome toxicities.

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PREDICTING HEMATOLOGIC CONSEQUENCES OF XENOBIOTIC EXPOSURES

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