

The Use of Hematological Effects in the Development of Minimal Risk Levels

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The Agency for Toxic Substances and Disease Registry (ATSDR) derives minimal risk levels (MRLs) to assist in evaluating risk of adverse health effects in individuals exposed to hazardous substances. MRLs are derived from published values identifying no-observed-adverse-effect levels (NOAELs) or lowest-observed-adverse-effect levels (LOAELs) in animal or human studies. The most sensitive end points are used. To date, 4 inhalation MRLs and 13 oral MRLs have been derived from hematological end points for 12 substances. This paper provides a brief overview of the hematological system, examples of hematological end points, and the MRL for substances with hematological end points.

INTRODUCTION

The Agency for Toxic Substances and Disease Registry (ATSDR) derives minimal risk levels (MRLs) to assist in evaluating risk of adverse health effects in individuals exposed to hazardous substances. The guidance for development of MRLs has been discussed elsewhere (Chou *et al.*, 1998). Substance-specific MRLs are discussed in the corresponding toxicological profiles developed by the agency. ATSDR toxicological profiles characterize the toxicologic and adverse health effect information for specific chemicals, by route and duration of exposure, and by health-effect end point.

The classification of end points is a critical component in the MRL derivation process. Adverse effects are effects that reduce the capacity of an organism or a component of an organism to function in a normal manner. The concept of adversity and its relationship to MRL derivation has been discussed elsewhere (ATSDR, 1995a; Chou *et al.*, 1998; Pohl and Abadin, 1995). ATSDR classifies the dose levels in relation to adverse health effects into three categories: no-observed-adverse-effect-level (NOAEL), lowest-observed-adverse-effect-level (LOAEL), and serious LOAEL (Chou *et al.*, 1998). By definition, LOAELs are dose levels at which observed health effects prevent an or-

gan or organ system from functioning in a normal manner over a lifetime, but will not necessarily lead to the inability of the whole organism to function normally. Serious LOAELs are dose levels at which the observed effects prevent the organism from engaging in normal activity over a lifetime. ATSDR does not derive MRLs from serious LOAELs. In addition, the ATSDR Minimal Risk Level Workgroup has adopted the classification of minimal LOAEL to take into account effect levels that reduce the capacity of an organ or organ system to function normally, but do not necessarily lead to the inability of the organ, organ system, or the whole organism to function normally.

This paper presents a brief overview of the hematological system and examples of adverse conditions. Specific examples of minimal risk levels derived from hematological end points are presented.

The Hematologic System

The hematopoietic system includes tissues and organs necessary for the generation, maturation, and destruction of blood cells, as well as the blood cells themselves. These tissues and organs include the mononuclear phagocyte system, spleen, lymph nodes, thymus, bone marrow, and liver.

Hematopoiesis is the term used to describe the production of blood cells. Three types of formed elements are found in the blood: red blood cells (RBCs) or erythrocytes, white blood cells (WBCs) or leukocytes, and platelets (thrombocytes). In healthy individuals, the destruction and production of cells are balanced, and the number of cells present in the blood at any specific time is fairly constant.

In the fetus, hematopoiesis takes place at various stages in the liver, spleen, thymus, bone marrow, and lymph nodes. Hematopoiesis begins as early as the 19th day after fertilization in the yolk sac of the human embryo with the production of primitive RBCs. When the fetal liver becomes the major site of blood cell production at approximately the 3rd month of development, the yolk sac ceases its function in hematopoiesis. Hematopoiesis also begins at this time in the spleen,

kidney, thymus, and lymph nodes. The lymph nodes serve as an important site of lymphopoiesis throughout life, whereas blood production in the liver, spleen, kidney, and thymus discontinues or decreases as the bone marrow becomes active in hematopoiesis. The bone marrow becomes the primary site of hematopoiesis at about the 6th month of development and continues as the primary source of blood production after birth and throughout life.

The erythrocyte is one of the most specialized cells in the body. Erythropoiesis is generally an orderly process through which the peripheral concentration of erythrocytes is maintained in a steady state. Membrane integrity is essential for normal erythrocyte function and survival. Inherited or acquired membrane abnormalities can lead to severe anemia. Since the mature RBC does not contain a nucleus, mitochondria, and other subcellular organelles, its metabolism is limited. The binding, transport, and release of CO_2 and O_2 are passive processes and do not require energy; however, a number of energy-dependent metabolic processes occur that are essential to the survival of the RBC. The most important metabolic pathways require glucose as a substrate. The function of erythrocytes is to transport oxygen from the lungs to the tissue and to remove carbon dioxide from the tissue to the lungs. The highly specialized protein contained in erythrocytes that is responsible for transport of these gasses is hemoglobin.

Leukocyte formation, following specific hormonal stimulation, is initiated in the bone marrow and results in various types of leukocytes: granulocytes (which include neutrophils, eosinophils, and basophils), monocytes, and lymphocytes. Upon maturation, these cells may be released into the peripheral blood, or they may remain in the bone marrow storage pool until needed. The defensive function of leukocytes includes two separate but interrelated events: phagocytosis and the subsequent development of the immune response. Granulocytes and monocytes are responsible for phagocytosis, while monocytes and lymphocytes interact to produce an effective immune response.

Platelets are also derived in the bone marrow and are involved in several aspects of hemostasis (blood coagulation). One of the major roles of the platelet appears to be that of passive surveillance of the blood vessel endothelial lining for gaps and breaks. In the event of an injury in which there is an actual break in the continuity of the lining of the vessels, the platelets react by forming an aggregate known as the primary hemostatic platelet plug and arrest bleeding. Another function of the platelet is the promotion of tissue repair following the injury. Hematological effects encompass effects to any of the components of the blood or blood-forming units. Although part of the hematological system, the thymus and spleen can be classified as immunologic due to their contribution to immunity.

Blood cell counts and various hematological measurements are typically used to evaluate hematotoxicity. These include, among others, erythrocyte count, thrombocyte count, and hematocrit and hemoglobin content. Effects on cell morphology and assessments of functional impairment of any component (e.g., heme synthesis) including hematopoiesis itself would constitute hematotoxicity. The following represents a list and brief description of some parameters and conditions used to evaluate hematotoxicity.

Potential End Points for MRL Derivation

Anemia. Anemia is a hematological condition that can result from various disease states including chemical exposure. Simply stated, anemia indicates a decreased amount of hemoglobin and, consequently, reduced oxygen-carrying capacity of the blood that may lead to hypoxia. If hypoxic conditions occur, important tissues (e.g., brain) may be affected due to oxygen deprivation. Diagnosis of anemia is partly made through the determination of red cell count, hemoglobin, and hematocrit. Morphologically, anemia can be classified according to average size and hemoglobin content of the circulating red blood cells. Red blood cell indices used to determine this include mean corpuscular volume or MCV, mean corpuscular hemoglobin or MCH, and mean corpuscular hemoglobin concentration or MCHC. These indices are often helpful since certain causes of anemia produce a characteristic morphology. Several classifications of anemia have been formulated. Although these classifications are important for the purpose of diagnosis and treatment, it is also important to note that a number of factors can complicate laboratory interpretation. These include multiple mechanism involvement or preexisting complicating factors such as iron deficiency. In addition, off-setting morphologic expression can occur. For example, iron deficiency typically results in microcytic red cells; with folate deficiency, red blood cells are usually macrocytic. In a combined iron and folate deficiency, one might observe normal MCV (McKenzie *et al.*, 1988).

There are three functional classifications of anemia: proliferative, maturation, and survival. Proliferative defects may be caused by inappropriate production of the hormone erythropoietin and are associated with malignancies, chronic renal disease, and certain endocrinopathies. The kidneys produce 80–90% of the body's erythropoietin (the liver is responsible for erythropoietin production in the fetus), which is the principal factor that stimulates RBC production. During hypoxia, erythropoietin production is increased, stimulating production of RBCs to alleviate the hypoxia. Chemically induced kidney damage can result in the interruption of the erythropoietic stimulating mechanism. Alternatively, this mechanism can be operating

correctly, but the bone marrow may not be responding to the stimulus because of chemical damage to the marrow. In both of these cases, anemic conditions result, but the underlying mechanism can be due to quite different compounds acting upon two different organ systems.

Maturation defects are those that are characterized by a disruption in the normal development of the RBC. The bone marrow usually recognizes the abnormal cells and destroys them before they reach the peripheral circulation. Reticulocyte levels in the circulating blood are, therefore, usually low. Cytoplasmic maturation defects are caused by abnormal hemoglobin production, and the defect is limited to the erythroid cell line (McKenzie *et al.*, 1988). Examples include thalassemia, hemoglobinopathies, lead intoxication, and porphyrias. Nuclear maturation defects affect all cell lines including erythrocytes.

Survival defects result from premature loss of erythrocytes by hemorrhage or hemolysis. A compensatory increase in bone marrow proliferation is observed as increased circulating reticulocytes. Other indicators include increased serum bilirubin, hemosiderinuria, hemoglobinuria, and hemoglobinemia (McKenzie *et al.*, 1988).

Methemoglobinemia. Methemoglobinemia refers to an increase in the amount of normal (2% of total hemoglobin) methemoglobin in the circulation. Methemoglobin contains ferric rather than ferrous iron and is not capable of combining with either oxygen or carbon dioxide. Blood appears greenish brown to black in color. Increased levels result in hypoxia (Fischbach, 1992). Common chemicals known to mediate the oxidation of hemoglobin to methemoglobin include aniline dyes, nitrates, nitrites, and chlorates. Methemoglobinemia is particularly important for infants in that fetal hemoglobin is more easily oxidized to methemoglobin than adult hemoglobin by, for example, drinking water containing high amounts of nitrites.

Heinz bodies. Heinz bodies are inclusions observed microscopically in RBCs that represent denatured and precipitated hemoglobin and may be associated with methemoglobinemia. These can result from exposure to oxidizing chemicals and will lead to premature RBC destruction in the spleen (McKenzie *et al.*, 1988). Hemosiderosis may be observed in various organs, particularly in the liver and the spleen.

2,3-Diphosphoglycerate (2,3-DPG). Synthesized and degraded by the RBC, 2,3-DPG regulates the affinity of hemoglobin for oxygen. The oxygen affinity of hemoglobin is inversely proportional to the concentration of 2,3-DPG. 2,3-DPG binds reversibly to hemoglobin, stabilizing the deoxy form (Smith, 1996). Increased levels are associated with hypoxia (from cardiac disease, anemia, and lung disease), uremia, and thyrotoxicosis.

Erythrocyte sedimentation rate (ESR). ESR reflects alterations in plasma proteins which cause clumping of RBCs and, therefore, a faster sediment rate. Increased values are associated with, among other things, inflammatory diseases, carcinoma, heavy metal poisoning, severe anemia, and nephritis (Fischbach, 1992).

Myoglobin. Myoglobin is the oxygen-binding protein for striated muscle. An increase in myoglobin concentration in the blood may indicate muscle damage.

Reticulocytosis. Reticulocytes are immature RBCs normally present in about 1% of circulating RBCs. They usually spend 2 or 3 days in the bone marrow before being released into the circulation, where they spend an additional 1 to 2 days before becoming mature erythrocytes (McKenzie *et al.*, 1988). An increase in the circulating reticulocytes (reticulocytosis) indicates an accelerated replacement of RBCs by the bone marrow and is a normal response to some types of anemic conditions (e.g., blood loss, hypoxia). Reticulocytosis can, therefore, be indicative of normal bone marrow activity and erythropoiesis. Conversely, decreased reticulocyte counts in the presence of anemia can indicate bone marrow failure (Fischbach, 1992). Reticulocytes are normally increased in pregnant women and in infants. Reticulocyte counts should be viewed in relation to the total number of erythrocytes in order to provide meaningful information.

Porphyrias. Porphyrias are a group of disorders that result from the disturbance in the heme biosynthetic pathway, resulting in accumulation of porphyrins in tissues and excretion of large amounts in the urine and/or feces. Heme synthesis occurs primarily in the bone marrow and liver leading to the formation of hemoglobin and microsomal P450 enzymes, respectively. Porphyrin disorders can, therefore, be separated into erythropoietic and hepatic, depending upon the site of biochemical and pathologic lesion (Fischbach, 1992; McKenzie, 1988). The basis for erythropoietic porphyrias is an alteration of one or more enzymes in the heme synthesis pathway.

MINIMAL RISK LEVELS

Seventeen minimal risk levels based upon hematological effects have been derived for 12 substances (see Table 1). The following are some examples.

Acetone. An intermediate-duration oral MRL of 2 mg/kg/day was derived for acetone based upon a 13-week study in rats (Dietz *et al.*, 1991; NTP, 1991). The MRL was derived from a NOAEL of 200 mg/kg/day; a LOAEL of 400 mg/kg/day produced evidence of macrocytic anemia in the male rat (ATSDR, 1994). The evidence consisted of a decrease in hemoglobin concentration, RBCs, reticulocyte counts, and platelets and an

TABLE 1
MRLs Based on Hematological Effects

Substance	Duration ^a	MRL	UF	End point used for MRL derivation	Primary reference
2-Butoxyethanol ^b	Acute	7	30	NOAEL: at higher dose, decreased RBC, MCHC; increased MCH, MCV	Tyl <i>et al.</i> (1984)
	Intermediate	3	30	NOAEL: at higher dose, decreased RBC, Hb; increased MCH	Dodd <i>et al.</i> (1983)
	Chronic	0.00004	1000	LOAEL: decreased Ht, Hb, RBC, reticulocyte; increased Mthb	Air Force (1985)
Propylene glycol dinitrate	Intermediate	0.00004	1000	LOAEL: decreased Ht, Hb, RBC, reticulocytes; increased Mthb	Air Force (1985)
	Chronic	0.00004	1000	LOAEL: decreased Ht, Hb, RBC, reticulocytes; increased Mthb	Air Force (1985)
Zinc	Intermediate	0.3	3	LOAEL: decreased superoxide dismutase activity, Ht, and serum ferritin	Yadrick <i>et al.</i> (1989)
	Chronic	0.3	3	LOAEL: decreased superoxide dismutase activity, Ht, and serum ferritin	Yadrick <i>et al.</i> (1989)
Acetone	Intermediate	2	100	NOAEL: at higher doses, mild microcytic anemia	NTP (1991)
Acrolein	Chronic	0.0005	100	NOAEL: at higher dose, increased monocytes	Long and Johnson (1989)
Acrylonitrile	Chronic	0.04	100	LOAEL: decreased RBC	Biodynamics (1980)
1,2-Dichloroethene	Acute	1	100	NOAEL: at higher dose, decreased Ht and RBC	McCauley <i>et al.</i> (1990)
	Intermediate	0.3	100	NOAEL: at higher dose, decreased Ht	McCauley <i>et al.</i> (1990)
2-Butoxyethanol ^b	Acute	0.4	90	LOAEL: hemoglobinuria	Ghanayem <i>et al.</i> (1987)
1,2-Dichloropropane	Intermediate	0.07	1000	LOAEL: slight anemia	Bruckner <i>et al.</i> (1989)
1,3-Dinitrobenzene	Intermediate	0.0005	1000	LOAEL: splenic hemosiderosis	Linder <i>et al.</i> (1986)
Diisopropylmethylphosphonate ^b	Intermediate	0.8	100	NOAEL: at higher doses, decreased Ht, RBC, Heinz body formation	Hart (1980)
2,6-Dinitrotoluene ^b	Intermediate	0.004	1000	LOAEL: cyanosis	Lane <i>et al.</i> (1985)
2,4-Dinitrotoluene ^b	Chronic	0.002	100	NOAEL: at higher dose, anemia	Ellis <i>et al.</i> (1979)

Note. Hb, hemoglobin; Ht, hematocrit; LOAEL, lowest-observed-adverse-effect level; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; mg/kg/day, milligram per kilogram of body weight per day; MRL, minimal risk level; Mthb, methemoglobin; NOAEL, no-observed-adverse-effect level; ppm, parts per million; RBC, red blood cell; UF, uncertainty factor.

^a By ATSDR definition: acute, 14 days or less; intermediate, 15–364 days; chronic, 365 days or more.

^b MRL subject to change pending release of final profile.

increase in MCV and MCH. Hemosiderin deposits in the spleen were also observed.

Propylene glycol dinitrate (PGDN). PGDN is the major component of Otto Fuel II, a torpedo propellant used by the U.S. Navy. A chronic and an intermediate-duration inhalation MRL of 0.00004 ppm was derived for PGDN based upon a study conducted in beagle dogs (ATSDR, 1995b). At a dose level of 0.2 ppm, hematological effects included decreased hematocrits, RBCs, hemoglobins, and reticulocytes and an increase in methemoglobin. Effects were first observed 4 weeks into the study period and persisted through to the end of the 14-month study period (Air Force, 1985). PGDN elicits hematological effects typical of those observed following exposure to nitrate/nitrite compounds.

Diisopropylmethylphosphonate (DIMP). ATSDR derived an intermediate-duration MRL of 0.8 mg/kg/day based upon a NOAEL of 75 mg/kg/day in beagle dogs (ATSDR, 1996). In this 3-month feeding study, no clear dose-related adverse hematological effects were observed at the highest dose tested (Hart, 1980). However, Heinz body formation, reticulocytosis, and decreased RBC and hematocrit have been observed in mink at higher doses (≥ 345 mg/kg/day) (Buchi *et al.*, 1992, 1994, 1997). These changes suggest that DIMP can cause oxidative damage to the hemoglobin, thus reducing survival time of the RBCs, which in turn induces hematopoiesis.

1,3-Dinitrobenzene. Splenic hemosiderosis was observed in rats exposed to 0.75 mg/kg/day of 1,3-dinitrobenzene for 12 weeks (Linder *et al.*, 1986). An intermediate-duration MRL of 0.0005 mg/kg/day was derived based upon this study (ATSDR, 1995c). The hematologic end points for this MRL are supported by other studies reporting increased erythropoietic activity, decreased hemoglobin, cyanosis, and hemosiderosis (Blackburn *et al.*, 1988; Cody *et al.*, 1981; Reader *et al.*, 1991). These effects are consistent with hemolytic anemia following exposure to nitroso compounds.

2,4- and 2,6-Dinitrotoluene (DNT). As with PGDN and 1,3-dinitrobenzene, the constellation of effects observed for 2,4- and 2,6-dinitrotoluene are common to nitroaromatic compounds as well as most organic and inorganic nitrates. For DNT, these include decreased RBCs, methemoglobinemia, Heinz body formation, hemosiderosis, and reticulocytosis (ATSDR, 1997). A chronic-duration MRL of 0.002 mg/kg/day was derived for 2,4-DNT based upon a 24-month dog study (Ellis *et al.*, 1979, 1985). Beagle dogs dosed once per day at 1.5 mg/kg/day developed methemoglobinemia and Heinz bodies. The MRL was based on the NOAEL of 0.2 mg/kg/day. An oral intermediate-duration MRL for 2,6-DNT was derived from another dog study by Lee *et al.* (1976). Animals administered 4 mg/kg/day of 2,6-DNT

were found to have extramedullary erythropoiesis and lymphoid depletion of the spleen.

CONCLUSION

Proper classification of health end points is a crucial element in the MRL development process. Oftentimes, the lines between NOAEL, LOAEL, minimal LOAEL, and serious LOAEL are not clearly defined. For hematological effects, the degree of response is also often very important. For example, normal levels of methemoglobin in humans vary (Fischbach, 1992). Doses causing methemoglobinemia may be considered minimal LOAELs if the response levels measured are within a normal range, and the database for the chemical in question supports this mechanism of toxicity. Doses causing higher levels of methemoglobin may, on the other hand, be considered serious if clinical signs of frank toxicity are observed.

Scientific judgement, coupled with the most current understanding of the chemical-specific mechanisms of action, must be used in cases such as these. Clearly then, it is important to understand not only the end point under consideration, but also the ramifications of these end points to overall human health.

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