UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: February, 2013

SUBJECT: A Retrospective Analysis of the Immunotoxicity Study (OCSPP Test Guideline No. 870.7800)

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In 2011, CropLife America (CLA) conducted a retrospective evaluation of 82 immunotoxicity studies to determine the potential regulatory impact of this study. The assessment concluded that the large majority of studies demonstrated no treatment-related effects at the high dose level for the given study and in no case was the immunotoxicity finding the most sensitive endpoint when compared to other existing toxicity endpoints in the data base, indicating the lack of impact of these studies on human health risk assessments. Based on their findings, the CLA requested that the Agency begin granting waivers for the immunotoxicity guideline study based on its authority under 40 CFR § 158.45 to grant waivers for studies for which "the data would not be useful in the Agency's evaluation of the risks or benefits of the product."

In 2012, the Office of Pesticide Programs (OPP) similarly conducted a retrospective analysis of over 170 immunotoxicity studies (including the 82 chemicals evaluated by CLA) for the purpose of 1) assessing if the results of the guideline immunotoxicity study impact human health risk assessment; 2) determining whether there is sufficient justification for granting waivers for this immunotoxicity study; and 3) proposing a path forward. The results of this analysis are presented in this document.

A Retrospective Analysis of Immunotoxicity Studies (870.7800)

Health Effects Division
Office of Pesticide Programs
U.S Environmental Protection Agency

February, 2013

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I. INTRODUCTION

In 2007 the 40 CFR Part 158 Toxicology Data requirements were revised to include an immunotoxicity study (870.7800). This study provides an evaluation of the function of immune systems components (*i.e.*, humoral or cell-mediated type immunity) which allow a better characterization of potential immunotoxicity for a test chemical.

The Office of Pesticide Programs (OPP) has now received and reviewed over 170 of these studies. Given that large number of these new immunotoxicity studies have now been submitted, OPP believes it is appropriate to conduct retrospective analysis for the purpose of 1) assessing if the results of the guideline immunotoxicity study impact human health risk assessment; 2) determining whether there is sufficient justification for granting waivers for this immunotoxicity study; and 3) proposing a path forward.

II. BACKGROUND

1. Toxicity Testing in General

OPP routinely requires extensive toxicity testing that evaluate a wide range of toxic effects for conventional pesticides as stipulated in 40 CFR Part §158 Toxicology Data requirements. These studies provide the scientific basis for characterizing the potential hazard of pesticide exposure. Toxicity studies are conducted in multiple species (mice, rats, rabbits, and dogs) for various durations (acute, subchronic and chronic) and evaluate various endpoints (nervous system, developmental-, reproduction, subchronic and chronic toxicity, immunotoxicity, carcinogenicity and mutagenicity). The Agency uses these studies to examine whether adverse effects are caused by different durations of pesticide exposure ranging from short-term (acute) to long-term (chronic) exposure and different routes of exposure (oral, dermal, or inhalation).

Toxicity studies allow the Agency to determine the level of exposure to the pesticide at which adverse effects might occur. An important aspect of this determination is assessing the relationship between exposure (dose) and response (often referred to as the dose-response analysis). The Agency then chooses values from a dose-response curve at the low end of the observable data (*i.e.*, the no observed adverse effect level, NOAEL and the lowest-observed adverse effect level, LOAEL, or a modeled benchmark dose) as a starting point for conducting a quantitative risk assessment. These values are referred to as the Points of Departure (POD). Separate PODs may be established for dietary (acute and chronic) and non-dietary pathways (incidental oral, dermal and inhalation) for various durations of exposure (short, intermediate and long term).

2. Immunotoxicity Testing in General

The immune system is responsible for modulating host defenses against a range of human diseases. The successful development and functioning of the immune system require recognition and response to a range of cellular and circulating signals acting by endocrine, autocrine, and paracrine mechanisms. These complex control systems offer multiple opportunities for disruption by environmental chemicals such as agricultural pesticides.

In 1993, the National Research Council (NRC) concluded that an analysis of the impact or toxicity of agricultural chemicals on the immune system is essential. Finding that there is a paucity of information on the effects of many chemicals on the developing and indeed on the mature immune system, the NRC recommended development of a battery of consensus tests to protect the developing immune system (NRC, 1993).

In 1996, the Agency's Science Advisory Panel (SAP) reviewed the draft guidelines for the immunotoxicity testing (OPPTS 870.7800) and agreed that the immune system is a legitimate target organ for toxicity. The SAP concluded that the OPP's proposed methodology was sufficiently validated in both the rat and mouse systems for inclusion into routine toxicology hazard evaluation studies. (SAP, 1996).

The European Union (EU) includes functional immunotoxicity testing assessment of the immune system in its 90-day rodent study and not as a core requirement. The Organization of Economic Co-operation and Development (OECD) make use of the Extended One-Generation Reproduction Study (OECD Test Guideline 443) to assess the potential impact of chemical exposure on the developing immune system *in lieu* of a guideline study.

3. EPA's Functional Immunotoxicity Study

Traditional subchronic and chronic rodent studies can provide some information on certain immunological endpoints such as hematology, lymphoid organ weights and histopathology. Nonetheless, EPA concluded in 2007 that these endpoints alone do not provide a full and integrated evaluation of immune function. Evaluating the potential of a chemical to adversely affect the immune system function is important because immune system suppression has been associated with increased incidences of diseases. Accordingly, that year the Agency revised the Part 158 Toxicology Data requirements to require a functional immunotoxicity study for all food and non-food use pesticides.

The Agency's immunotoxicity study (OPPTS. 870.7800) is designed to evaluate the immunosuppressive potential of a chemical by measuring antibody production to sheep red blood cells (SRBCs), a T-dependent antigen, in mice or rats. The T-cell dependent antibody response (TDAR) is dependent on functional T-cells, B-cells and antigen processing/presenting cells and is considered a reliable indicator for measuring humoral immune function (USEPA, 2008, 2009).

In the immunotoxicity study, mice and/or rats are exposed orally (gavage or diet) or via inhalation to the test and control substances for at least 28 days. Four or 5 days prior to the end of exposure (*e.g.*, day 24 or 23 of the study) animals are immunized by intravenous or intraperitoneal injection of SRBCs. The day of immunization depends on whether antibody

forming cells (day 23) or antibody titers (day 24) are assessed. Animals are sacrificed on Day 29. At the end of the exposure period, either the plaque forming cell assay (PFCA) or an enzyme linked immunosorbent assay (ELISA) is performed to determine TDAR through evaluation of the effects on the number of splenic anti-SRBC producing cells or serum anti-SRBC IgM titers, respectively. Other parameters evaluated in the study include mortality, clinical signs of toxicity, body weight, body weight gain, food consumption, water consumption, organ weights (i.e. spleen and thymus), and macroscopic pathology.

Depending on the outcome of the anti-SRBC assay, it may be necessary to perform a quantitative analysis of the effects of a chemical on the numbers of cells in major lymphocyte populations and T cell subpopulations by flow cytometry, or a splenic NK cell activity assay to assess the effects of the test compound on non-specific (innate) immunity

4. Non-EPA Retrospective Analysis

CropLife America conducted a retrospective evaluation of 82 TDAR studies to determine the potential regulatory impact of the immunotoxicity study. The assessment concluded that the large majority of studies demonstrated no treatment-related effects on TDAR at the high dose level for the given study. A reduction in TDAR was observed in 4 of the 82 studies at the high-dose level only; however, in no case was the immunotoxicity finding the most sensitive endpoint when compared to other existing toxicity endpoints in the data base, indicating the lack of impact of these studies on human health risk assessments (CLA, 2011, 2012).

OPP is now in position to complete a more comprehensive retrospective than the industry evaluation.

II. METHODS

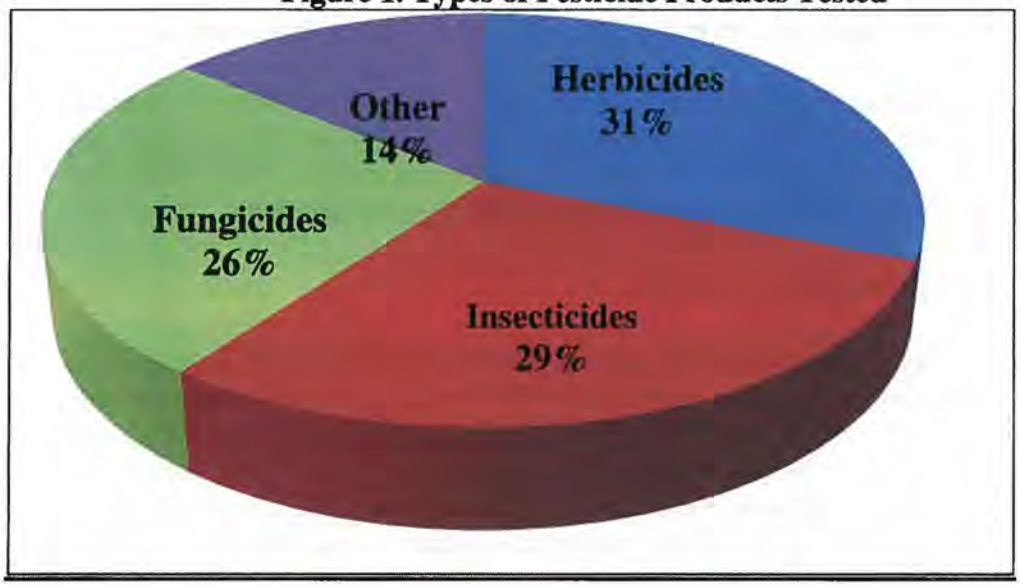
A. <u>Studies Evaluated</u>: The data set included 171 immunotoxicity studies conducted on 155 chemicals representing a range of product types and chemical structures including older and newer active ingredients. HED conducted an independent review of the studies and prepared Data Evaluation Records (DERs). The DER is the official record of independent review with sufficient detail to support study conclusions and contains the data review and analysis of study results. Only guideline acceptable studies were considered in this analysis.

	Total	**Rat**	**Mouse**	**Both Species**
Number of Chemicals Tested	155	83	58	14
Number of Studies Conducted	**Total**	**Males**	**Females**	**Both Sexes**
Rats	97	30	40	27
Mice	74	12	51*	11

* Two chemicals were evaluated in more than one study

B. Pesticide Product Types: This retrospective analysis data set represented a wide distribution of pesticide product types that included: herbicides (31%); insecticides (29%); fungicides (26%) and a small number of other product types (14%) such as acaricides, fumigants, plant growth regulators, bactericides, and biocides (Figure 1).

Figure 1. Types of Pesticide Products Tested



C. Pesticide Chemical Class: The pesticide chemicals tested for immunotoxicity in this data set represent a variety of chemical classes. Table 1 below presents the primary classification for the chemicals included in this analysis.

Table 1. Classification of Chemicals Tested in the Immunotoxicity Studies

Chemical Class	No. Chemicals	Chemical Class	No of Chemicals
Amide Fungicide	10	Nicotinoid Insecticide	4
Amide Herbicide	9	Nitrile Herbicide	1
Antibiotic Fungicide	1	N-Methyl Carbamate Insecticide	1
Arcaricide	1	Organophosphates	20
Arl Phenyl Ketone Fungicide	1	Oxadiazine Insecticide	1
Benzoic acid Herbicide	1	Oxazole Herbicide	2
Benzothiadiadiazole Fungicide	1	Phenoxy Herbicide	2
Benzoylcyclohexanedione Herbicide	1	Phenoxypropionic Herbicide	1
Benzoylurea Insecticide	2	Phenyl ether	1
Biologicals	1	Plant Growth Regulator	2
Bridged Diphenyl Acaricide	1	Polycyclic Aromatic Hydrocarbon	1
Carbazate Acaricide	1	Polynuclear Aromatic Hydrocarbon	1
Chitin Synthesis Inhibitor	1	Pyrazoles	7
Conazole Fungicide	8	Pyrethroids	8
Cyclohexene Oxime Herbicide	1	Pyridines	6

Table 1. Classification of Chemicals Tested in the Immunotoxicity Studies			
Chemical Class	**No. Chemicals**	**Chemical Class**	**No of Chemicals**
Diacylhydrazine Insecticide	1	Pyrrole Fungicide	1
Diamide Insecticide	1	Quaternary Ammonium	2
Dicarboximide Fungicide	2	Quinazoline Insecticide	1
Dicarboximide Herbicide	1	Quinoline Fungicide	1
Dinitroaniline Herbicide	1	Quinolinecarboxylic Herbicide	1
Diphenyl ether Herbicide	1	Spinosyn Insecticide	1
Dithiocarbonate Fungicide	2	Strobilurin Fungicide	2
Fumigants	3	Sulfonylurea Herbicide	6
Fungicide, unclassified	2	Tetramic acid Insecticide	1
Herbicide, unclassified	1	Tetronic acid Insecticide	1
Herbicide Safener	3	Thiazole Fungicide	1
Imidazoles	2	Thiazole Insecticide	1
Imidazolinone Herbicide	2	Thiazolidine Acaricide	1
Inorganic	1	Thiocyanate	1
Insecticide, unclassified	3	Triazine Herbicide	3
Microbiocide	1	Triazolone Herbicide	4
Morpholine Fungicide	1	Triazolopyrimidine Fungicide	1

D. Species Tested

Acquiring meaningful immunotoxicology data is greatly influenced by the selection of the appropriate species since one species may differ in sensitivity. The Agency recommended that the selection of the appropriate species (mouse or rat) and sex should be based on data from the existing Part 158 toxicity studies. In a weight of evidence approach any indicators for potential immunotoxicity (*i.e.*, changes in hematology, thymus or spleen weights and/or histopathology) were considered first, followed by data on general toxicity (*i.e.*, body weight, body weight gain, target organ toxicity) and all available toxicity information. In the absence of such data, the mouse was selected as the default species of choice for immunotoxicity testing. This decision was based on experience and the available historical control data on immunotoxicity in the mouse (USEPA, 2008).

Although the mouse was recommended by the Agency as the default species of choice, this data set includes more chemicals that were tested in rats (83/155 chemicals; 54%) than mice (58/155 chemicals; 37%); only 14 (9%) chemicals were tested in both species (Figure 2).

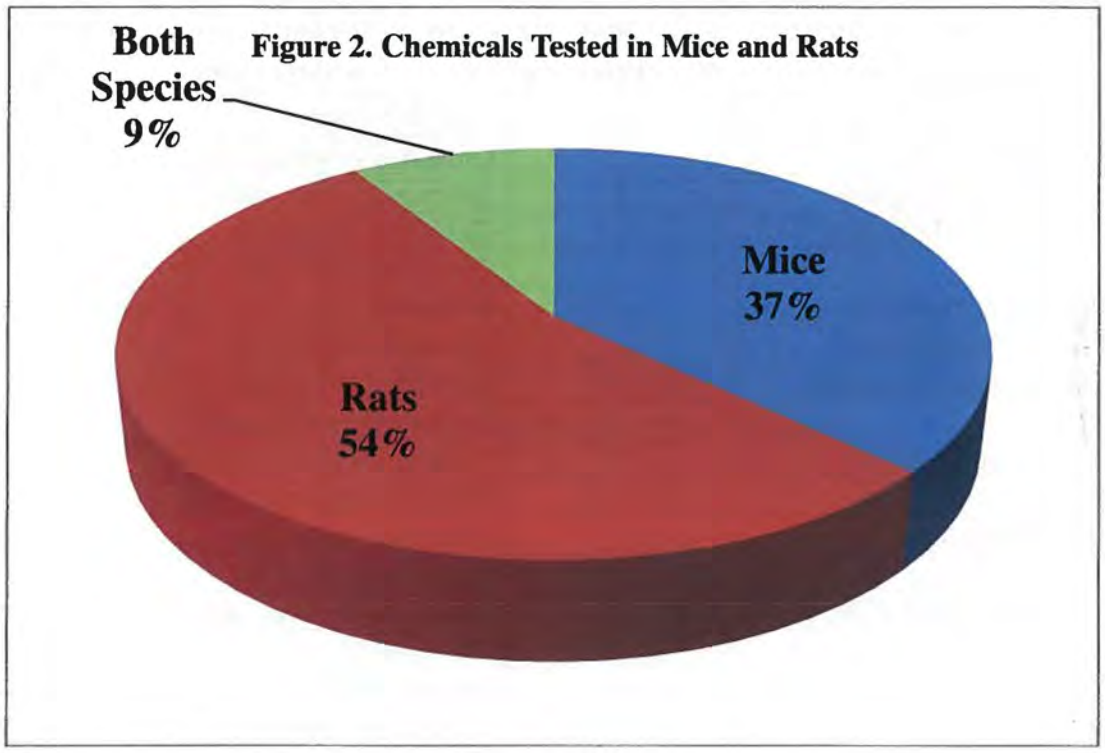
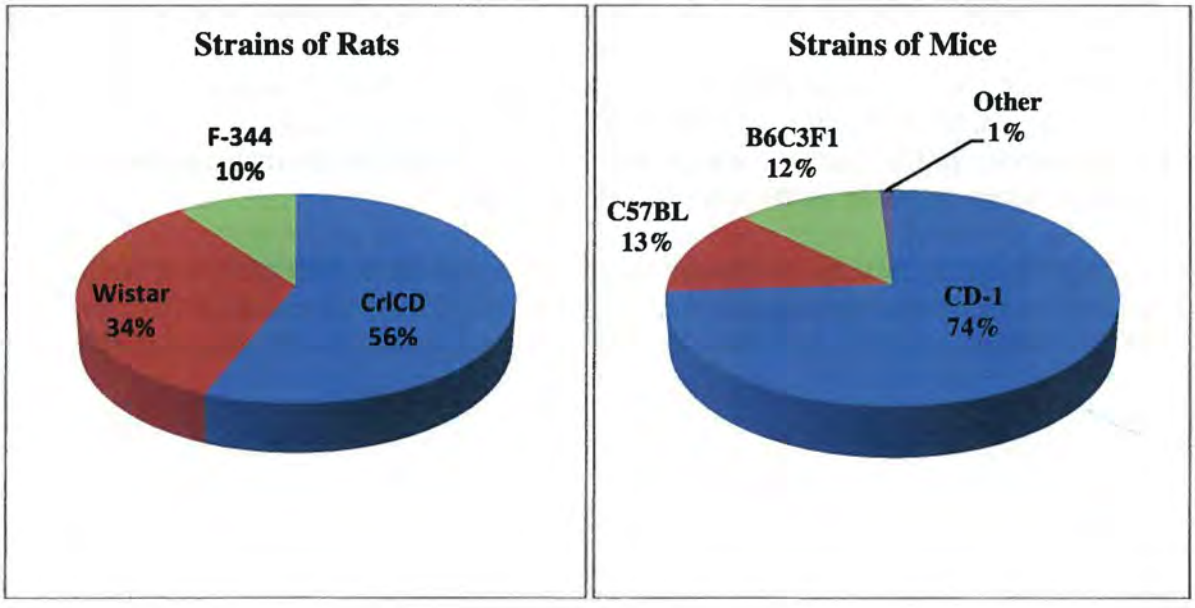


Figure 2. Chemicals Tested in Mice and Rats

A number of different strains of mice and rats were represented in this data set. Among the rats, the majority of the chemicals were tested in Crl:CD Sprague-Dawley (56%) or Wistar (34%) stocks. Among mice, the majority of the chemicals were tested in the CD-1 strain (74%) (Figure 3).

Figure 3. Species and Strains Tested



E. Sex Tested

The Agency recommended the use of the females as the sex of choice for the immunotoxicity study. Consistent with this recommendation this data set represents more studies conducted in females (69%, mice and 41%, rats studies). Also, of the rat studies, 31% were conducted with males only, and 28% used both males and females. Of the mouse studies, 16% used males only, and 15% used both sexes.

The data set included 171 studies with 97 in rats and 74 in mice. Of the 97 studies in rats, 30 were conducted in males, 40 in females and 27 in both sexes. Of the 74 studies in mice, 12 were conducted in males, 51 in females and 11 in both sexes (Figures 4 and 5).

Figure 4. TDAR Assays Conducted in Rats

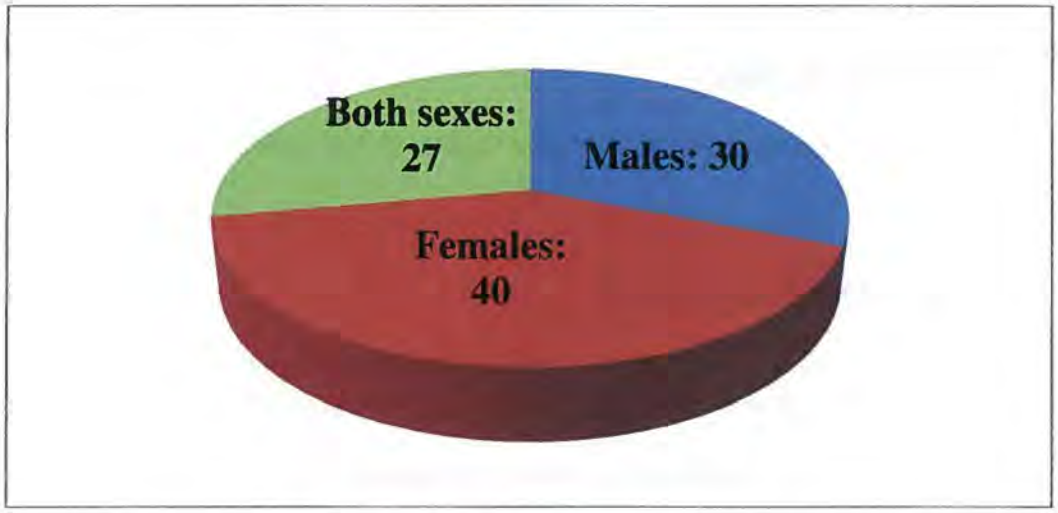


Figure 5. TDAR Assays Conducted in Mice

F. Routes of Administration

Of the 171 studies, the test material was administered via the diet in 89 studies to rats and in 66 studies to mice; via oral gavage in 7 studies to rats and in 5 studies to mice; and via inhalation in 4 studies in rats/mice.

G. Assay Methodology

1. TDAR assay

Of the 155 chemicals tested, 83 chemicals assessed TDAR using the ELISA method while 72 chemicals used the PFCA method, indicating that both accepted approaches were well-represented in the data set.

2. NK cell activity assay

The NK cell activity assay (innate immune response) was conducted for 11 chemicals.

III. RESULTS

A. Evidence of Immunotoxicity

There was no evidence of immunotoxicity for 140 (90%) of the 155 chemicals when evaluated for effects on the anti-SRBC IgM antibody response using the TDAR assay.

The 15 chemicals (10%) where immunotoxicity was seen are presented in Table 2.

Table 2. Chemicals that demonstrated immunotoxicity		
Amicarbazone	Fenamidone	Penthiopyrad
Boric acid	Fluazinam	Pyraflufen-ethyl
Buprofezin	Fomesafen	Thiram
Cyflufenamide	Imazosulfuron	Trichlorfon
Difenoconazole	Novaluron	Triflumizole

In terms of how these positive results were distributed between rat and mouse studies, immunosuppression was seen in 8 of 58 chemicals (14%) tested in mice and in 7 of 83 chemicals (8%) when tested in rats. A species difference was seen for two chemicals, Cyflufenamide and Penthiopyrad. When tested at the Limit Dose in both species, Cyflufenamide exhibited immunosuppression in rats, but not in mice while Penthiopyrad exhibited immunosuppression in mice, but not in rats.

There was no effect of NK cell activity for the 11 chemicals tested for natural killer (NK cell) activity (innate immune response). This set included the chemical triflumizole that showed reduced anti-SRBC IgM production in the TDAR assay.

B. Sensitivity of Immunotoxicity:

As shown in Table 3, for 9 of the 15 chemicals where immunotoxicity was observed by the TDAR assay, immunotoxicity was not more sensitive than systemic toxicity endpoints in those studies.

- For 4 chemicals (*Amicarbazone, Difenoconazole, Fenamidone, and Imazosulfuron*) immunotoxicity was seen at the same dose at which systemic toxicity occurred.
- For 3 chemicals (*Buprofezin, Cyflufenamide and Pyraflufen-ethyl*), tested in both sexes, immunotoxicity was seen only in one sex and at the same dose that caused systemic toxicity.
- For 2 chemicals (*Fomesafen, Penthiopyrad*) immunotoxicity was seen at a higher dose than the systemic toxicity dose.

Table 3 Immunotoxicity Seen in the Presence of Systemic Toxicity in the Study

Chemical	Immunotoxicity NOAEL (mg/kg/day)		Immunotoxicity LOAEL (mg/kg/day)		Systemic Toxicity NOAEL (mg/kg/day)		Systemic Toxicity LOAEL (mg/kg/day)	
Amicarbazone	81		195		81		195	
Buprofezin	M: 343	F:79	M: NE	F: 346	M:79	F: 79	M: 346	F:346
Cyflufenamide	M: 942	F:150	M: NE	F: 927	M: 927	F: 150	M: NE	F: 927
Difenoconazole	35		177		35		177	
Fenamidone	118		387		118		388	
Fomesafen	16		176		NE		16	
Imazosulfuron	222		710		222		710	
Penthiopyrad	301		1136		75		301	
Pyraflufen-ethyl	M: 236	F: 1114	M: 946	F: NE	M: 236	F: 1114	M: 946	F: NE

M= Male; F=Female; NE= Not Established

As shown in Table 4, 6 chemicals showed immunotoxicity in the TDAR assay in the absence of systemic toxicity in the study. However, as discussed below, when the immunotoxic dose levels were compared to the toxicology data base of these chemicals, immunotoxicity was not the most sensitive endpoint.

Table 4. Immunotoxicity Seen in the Absence of Systemic Toxicity in the Study				
Chemical	**Immunotoxicity NOAEL** (mg/kg/day)	**Immunotoxicity LOAEL** (mg/kg/day)	**Systemic Toxicity NOAEL** (mg/kg/day)	**Systemic Toxicity LOAEL** (mg/kg/day)
Boric acid	250	500	1000	NE
Fluazinam	11	231	1684[a]	NE
Novaluron	200	2115	2115	NE
Thiram	25	74	74	NE
Trichlorfon	32	164	164	NE
Triflumizole	29	286	286	NE

a = this was the highest dose tested in the first study. NE= Not Established

Boric acid: Two separate immunotoxicity studies were conducted. In the first study, immunotoxicity was seen when tested only at the Limit Dose; the TDAR LOAEL was 1000 mg/kg/day *(MRID No.48786601)*. In the follow-up study, boric acid was tested at dose levels of 250, 500, 750 or 1000 mg/kg/day. The TDAR NOAEL was 250 mg/kg/day, the lowest dose tested *(MRID No. 48798001)*. No spleen or thymus effects were seen in either study. No systemic toxicity was seen in either study. In the existing repeated dose studies, testicular effects were the most sensitive endpoint. The LOAEL for testicular effects is 32 mg/kg/day in the subchronic dog study while the TDAR LOAEL is 500 mg/kg/day in mice.

Fluazinam: Two separate immunotoxicity studies were conducted. In the first study, immunotoxicity was seen at all 3 doses tested; a TDAR NOAEL was not established *(MRID No. 48708702)*. In the second study, doses tested were lower than those used in the first study. The TDAR NOAEL was lower than the highest dose tested (HDT); the LOAEL was the HDT. In both studies, suppression of TDAR was seen in the absence of any systemic toxicity *(MRID No. 48708703)*. No spleen or thymus effects were seen in either study. In the existing repeated dose studies, hepatotoxicity was the most sensitive endpoint. The LOAEL for liver effects seen is 10 mg/kg/day in the mouse carcinogenicity study while the TDAR LOAEL is 231 mg/kg/day in mice.

Novaluron: The TDAR NOAEL was lower than the HDT; the LOAEL was the HDT. Increases in absolute and relative spleen weights were seen at the high dose. No thymus effects were seen. In the existing repeated dose studies, hematotoxicity was the most sensitive endpoint. The LOAEL for extra medullary hematopoiesis (EMH) and hemosiderosis in the subchronic rat is 9 mg/kg/day while the TDAR LOAEL is 2115 mg/kg/day in mice.

Thiram: The TDAR NOAEL was lower than the HDT; the LOAEL was the HDT. Increases in absolute and relative spleen weights were seen at the mid and high dose groups. No thymus effects were seen. Liver, blood and urinary system are the target organs for this chemical. In the existing studies, the LOAEL for changes in hematology, clinical chemistry, and urinary parameters, increased incidence of bile duct hyperplasia, and reduction in body weight gain seen in the rat carcinogenesis study is 7 mg/kg/day while the TDAR LOAEL is 74 mg/kg/day in mice.

Trichlorfon: The TDAR NOAEL was lower than the HDT; the LOAEL was the HDT. No spleen or thymus effects were seen. However, cholinesterase activity was not measured in this study. Based on the available data in the repeated studies, systemic NOAEL/LOAEL would have been established if cholinesterase activity would have been measured. The LOAEL for brain cholinesterase inhibition in monkeys is 1.0 mg/kg/day while the TDAR LOAEL is 164 mg/kg/day in rats.

Triflumizole: Both TDAR and a NK cell activity assays were conducted for this chemical. Immunosuppression was seen in the TDAR assay, but not in the NK cell activity assay. The TDAR NOAEL was lower than the HDT; the LOAEL was the HDT. No spleen or thymus effects were seen. Suppression of TDAR was seen in the absence of any systemic toxicity. In the existing repeated dose studies, hepatotoxicity was the most sensitive endpoint. The LOAEL for liver effects seen in the chronic dog is 34 mg/kg/day while the TDAR LOAEL is 286 mg/kg/day in mice.

C. Indicators of Immune System Endpoints

As shown in Table 5 below, indicators of potential immune toxicity are derived from routine measurements and examinations performed in toxicity studies submitted under Part 158 Toxicology Data requirements. The immune-related endpoints that are evaluated in these studies are presented below:

Table 5. Potential Immune System Endpoints

Study Type	Hematology	Spleen weight	Thymus weight	Spleen histopath.*	Thymus histopath.*	Lymph node histopath.*
Oral 28-day Rodent	x	x	x	x	x	x
Oral 90-day Rodent	x	x	x	x	x	x
Oral 90-day Non-Rodent	x	x	x	x	x	x
Dermal 21/28 day	x	x	x	x	x	x
Reproduction and Fertility		x (P, F1,F2)	x (F1, F2)	if target organ	if target organ	
Chronic toxicity	x	x		x		x
Chronic Toxicity/ Carcinogenicity	x	x		x		x

*Histopathological evaluation required on all control and high dose groups followed by mid-and low-dose groups if treatment-related findings are seen in the high dose.

1. **Clinical Pathology:** Hematologic parameters recommended in toxicity studies are generally the same as those performed clinically as human and animal health screens. Alterations in certain hematological parameters, such as decreases in hemoglobin (HGB), hematocrit (HCT), and red blood cell counts (RBC) are indicative of increased loss or decreased production of erythrocytes. When observed alone (*i.e.*, without concurrent white blood cell (WBC) or leukocyte changes), these erythrocyte changes are not typically associated with immunosupression. However, absolute and differential WBC counts and albumin/globulin (A/G) ratio may provide some indication of potential immunotoxicity. For example, changes in leukocyte counts may indicate altered bone marrow function and the potential for decreased production of immune cell precursors or products (*e.g.*, immunoglobulins). A shift in the A/G ratios may signal altered antibody synthesis. These data alone, however, are generally not considered to be specific indicators of direct immunotoxicity.

2. **Organ Weight:** In a toxicity study, organ weight changes provide the first signs of treatment-related changes in target organs during treatment. Changes in organ weights (absolute and relative weights) on specific immune organs *(e,g.,* thymus or spleen) provide useful information indicative of potential immunotoxicity. Since body weight is a co-variant in calculating a relative organ weight, elevated or depressed body weights should be considered when interpreting potential effects on immune organs.

   The thymus is a primary lymphoid organ where most T cells mature and decreased thymus weight may indicate potential immunotoxicity following exposure to immunotoxic chemicals. However, the thymus gland grows rapidly in young animals and begins to involute as the animal reach maturity; therefore it may be difficult to detect and measure this end point in adult and very old animals. Also, thymic weight may be highly variable resulting from normal biological variation as well as tissue collection techniques. Evidence of atrophy of the thymus observed in a short-term study (28/ 90 days) could be an indicator of immunotoxicity. On the other hand atrophy of the thymus in a chronic study may be attributable to the age of the animal rather than to frank immunotoxicity.

   The spleen is a secondary lymphoid organ where T-cells and B-cells mature. The spleen is also involved in limited erythropoiesis (production of red blood cells), B- and T-cell clonal expansion and serves as a biological filter for circulating blood. Increased spleen weights may result from a variety of causes, including increased hematopoiesis, increased processing of damaged erythrocytes, congestion, and neoplasia.

   Organ weight data alone or in relation to body weights can be sensitive measures of organ atrophy, hyperplasia, or hypertrophy, but yield little information about immunotoxic effects. In general changes in organ weight (thymus and spleen) should not be considered as a standalone measure to determine immunotoxicity because it may also indicate generalized systemic toxicity or indirect immunotoxic effects. In practice, however, changes in organ weights or organ-to-body-weight ratio are more relevant to immunotoxicity when they are associated with appropriate histopathological findings.

3. **Histopathology:** The immune system is composed of diverse groups of organs and tissues including those responsible for immune cell hematopoiesis (*e.g.*, bone marrow, spleen), lymphocyte maturation (thymus) and immune surveillance (*e.g.*, lymph nodes (Van Rees et al., 1996; Travlos, 2006)). Gross and microscopic examination of lymphoid tissues (*i.e.*, thymus, spleen, lymph nodes) is part of routine toxicity studies and morphologic changes resulting from immune cell toxicity in these tissues are described elsewhere (Ward *et al.*, 1999; Suttie, 2006; Maronpot, 2006; Kuper, 2006, Elmore, 2006, 2012). Changes in lymphoid organs histopathology (*e.g.*, thymus atrophy, lymphoid necrosis) may represent either a direct immunotoxic effect or a secondary effect related to a primary non-immunotoxic outcome. Histopathological indicators should thus be evaluated in the context of other findings.

   For example, hemosiderin deposits of the spleen may occur secondary to hemorrhage or hemolysis that is unrelated to direct immunotoxicity. Similarly, spleen congestion (passive accumulation of blood) may result from non-immunotoxic effects such as hemolytic anemia or barbiturate euthanasia. On the other hand, histological changes in the thymus, spleen, and lymph nodes at doses lower than those eliciting frank systemic toxicity may reflect deficits in immune function not typically assessed in other available Part158 studies. Also, if the test compound is shown to either stimulate cell proliferation or to cause atrophy and cell depletion in any lymphoid organ without clear evidence for a primary non-immunotoxic etiology, the effect is likely to be viewed as a potential indicator of an immunotoxic effect.

D. Indicator of Potential Immune Toxicity

There were no indicators of potential immunotoxicity in the existing toxicology data base for 125 of the 155 chemicals (81%) included in this data set. Observational immune system endpoints seen in the remaining 30 chemicals (19%) included: alterations in hematology parameters; changes in spleen weights; and/or histopathological lesions of the spleen and the thymus.

The endpoints evaluated in the Part 158 toxicity studies provide valuable insight in determining the potential immunotoxicity of the subject chemical, particularly when evaluated in a weight of evidence context (*i.e.*, examination of multiple immune-related parameters across multiple studies, using multiple test species and multiple exposure scenarios). Therefore, when interpreting the relevance of any potential immune-related findings, biological relevance, plausibility, dose-responsiveness, consistency of findings, corroborative endpoints, background levels, and statistical significance, etc. must also be carefully considered. In addition, it may be instructive to consider a finding of questionable significance in light of relevant historical control data in studies of similar duration with animals of the same species and strain. Historical control data may be used to identify aberrant concurrent control values, in order to determine if the concurrent control group is consistent with the larger population of controls. Because the immune system has been shown to be sensitive to stress, care should be taken in interpreting immune effects that are observed only in the presence of overt general toxicities as such effects may not be primary, but rather secondary in nature. If changes to immune endpoints are seen, it should be considered whether these effects may be secondary to general toxicity rather than toxicity on the immune system.

1. Immune Endpoint Indicators in Chemicals with Positive TDAR:

In the existing repeated dose studies, potential indicators of effects on the immune system were seen only for 4 of 15 chemicals that showed immunotoxicity. The findings are discussed below:

Amicarbazone: Subclinical anemia and hemosiderin pigmentation of the spleen was seen only at 709 mg/kg/day, the highest dose tested (HDT) in the carcinogenicity study in mice. Hemosiderin is normally present in the spleen as a storage form of iron and tends to increase with age in both rodents and dogs (Cesta, 2006). It may indicate prior hemorrhage/hemolysis but does not directly relate to immunotoxicity (Suttie, 2006). In this case, it was seen in older animals (sacrificed at week 78).

Boric acid: Decreased HGB and HCT levels and increased hemosiderin pigment in spleen were observed in the subchronic study in dogs. These hematological changes are consistent with anemia. As noted above, hemosiderin is normally present in the spleen and does not directly relate to immunotoxicity.

Fomesafen: Decreased RBC, HGB, MCV, and MCHC levels only at a very high dose (1247 mg/kg/day) in a 28-day study in mice and decreased HGB, HCT levels and RBC, and increased platelet count and prothrombine time only at the HDT (25 mg/kg/day) in a 26 week study in dogs. These findings are consistent with anemia and blood clotting time but not to immunotoxicity.

Novaluron: This chemical primarily produced red blood cell effects that are associated with compensatory erythropoiesis in mice, rat and dogs. Splenic findings were associated with the primary erythroid toxicity of novaluron, which caused secondary accumulation of damaged erythrocytes in the spleen following removal from the blood. Increased spleen weights and/or hemosiderosis in the spleen are considered to be secondary to processing damaged erythrocytes and not to be an immunotoxic effect. Similarly, increased extramedullary hematopoiesis may represent an adaptive response.

2. Immune Endpoint Indicators in Chemicals with Negative TDAR

Changes were noted in immune endpoints in the existing repeat dose studies for 26 of the 140 chemicals that showed no effect on TDAR at any dose level. The potential immune related effects observed included alterations in hematology parameters, changes in spleen weight, and/or histopathological changes in the spleen and thymus.

Alterations in **hematology** parameters were the sole findings for 12 chemicals. These alterations could be viewed as potential indicators of immunotoxicity; however,in these incidences they were considered to be indicative of anemia and not indicators of immunotoxicity. Most of the changes in blood parameters are related to anemia. The changes seen in WBC parameters for five of these chemicals were determined to be not toxicologically relevant changes since these findings were seen in isolation, in the absence of dose or time concordance and were not corroborated with any other findings such as changes in lymphoid organs, bone marrow damage or histopathological lesions in the lymphoid organs.

Changes in **spleen weights and/or histopathological** alterations were observed only for three chemical and are unlikely to be indicative of immunotoxicity. Splenic function is centered on the systemic circulation removing foreign material and damaged cells from the blood. It also serves as a repository for iron, erythrocytes, and platelets. In rodents, it is a site of hematopoiesis – more so in mice than in rats - particularly in fetal and neonatal animals (Cesta, 2006). In contrast, hematopoietic tissue is present in the spleen of dogs in pathologic conditions such as neoplasia and anemia, but may be present in the absence of underlying disease (HoganEsch and Hahn, 2001). Since extramedullary hematopoiesis is commonly seen in rodents (particularly mice) in the absence of underlying disease and can be indicative of anemia/ hemorrhage/hemolysis, systemic inflammation, splenic trauma/necrosis, decreased hematopoiesis by the bone marrow, and neoplasia; spleen histopathological changes in the absence of other lesions are unlikely to be specific indicators of immunotoxicity.

Changes in **thymus** were seen only in two chemicals and were characterized as atrophy of the thymus for one chemical and lymphocytolysis in the other. The histopathological changes of the thymus observed with these two chemicals are potentially secondary to stress and not specific indicators of immunotoxicity.

A mixture of these effects (alterations in hematology parameter, changes in spleen weight and histopathological lesions in the spleen) were seen for 13 chemicals.

Overall analysis of the other endpoints in the toxicology database (*i.e*, clinical pathology, organ weight and histopathology data) indicate that these findings are generally not reliable or sensitive indicators of potential immunotoxicity in these studies.

E. Impact of Immunotoxicity Study on Human Health Risk Assessment

A seminal question raised in this analysis is whether the immunotoxicity study has the potential to yield a more sensitive endpoint or a lower Point of Departure (POD) that would replace an existing POD selected for various scenarios in human health risk assessment. Consequently, the ratio of the NOAEL/LOAEL for TDAR was compared to the PODs selected for various exposure scenarios.

Of the 155 test chemicals in this analysis, 140 were reported to cause no effect on the TDAR up to the highest dose tested (*i.e.*, no evidence of immunotoxicity).

Of the 15 chemicals that showed TDAR, the TDAR NOAELs and LOAELs were considerably greater than the PODs selected for various risk assessment scenarios except for Imazosulfuron for which the TDAR NOAEL was numerically comparable to the POD selected for non-dietary (i.e, incidental oral and inhalation) exposure scenarios. The perceived equivalency could be an artifact of dose selection since the LOAEL was 3-fold higher than the POD.

Table 6 compares the ratio of the TDAR NOAELs and LOAELs to the various PODs selected for short and intermediate-term non-dietary (incidental oral, dermal, and inhalation) and chronic dietary (chronic RfD) exposure scenarios.

Table 6. Impact of Immunotoxicity On Existing Risk Assessment

Exposure Scenario	Points of Departure (POD) (mg/kg/day)	Immunotoxicity NOAEL/LOAEL (mg/kg/day)			Magnitude Higher (x-fold) than POD to			
					NOAEL		LOAEL	
Amicarbazone – Female Rat								
S/I -Incidental Oral	6.28	NOAEL = 81 LOAEL = 195			13		30	
-Dermal	Assessment not required				Not Applicable			
-Inhalation	6.28				13		30	
Chronic Dietary	2.30				35		85	
Boric acid – Female Mouse								
S/I -Incidental Oral	8.80	NOAEL =250 LOAEL= 500			28		57	
-Dermal	Assessment not required				Not Applicable			
-Inhalation	8.80				28		57	
Chronic Dietary	Not Required				Not Applicable			
Buprofezin – Male / Female Rat								
S/I -Incidental Oral	Assessment not required		M	F	Not Applicable			
-Dermal	Dermal NOAEL 300	NOAEL	343	79	See foot note [a]			
-Inhalation	4.3 [b]	LOAEL	NE	346	M: 80	F: 18	M: NE	F:80
Chronic Dietary	1.0				M:343	F: 79	M:NE	F:346
Cyflufenamid – Male / Female Rat								
S/I -Incidental Oral	5.0		M	F	M:188	F:30	M:NE	F:185
-Dermal	Assessment not required	NOAEL	942	150	Not Applicable			
-Inhalation	5.0	LOAEL	NE	927	M:188	F:30	M:NE	F:185
Chronic Dietary	4.40				M:214	F:38	M:NE	F:21
Difenoconazole – Female Mouse								
S/I -Incidental Oral	1.25	NOAEL = 35 LOAEL = 177			28		142	
-Dermal	1.25				28		142	
-Inhalation	1.25				28		142	
Chronic Dietary	0.96				36		184	
Fenamidone – Male Rat								
S/I -Incidental Oral	10.4	NOAEL = 118 LOAEL = 387			11		37	
-Dermal	10.4				11		37	
-Inhalation	10.4				11		37	
Chronic Dietary	2.83				42		137	

Table 6. Impact of Immunotoxicity On Existing Risk Assessment

Exposure Scenario	Points of Departure (POD) (mg/kg/day)	Immunotoxicity NOAEL/LOAEL (mg/kg/day)	Magnitude Higher (x-fold) than POD to	
			NOAEL	LOAEL
Fluazinam – Female Mouse				
S/I -Incidental Oral	4.0	NOAEL = 11 LOAEL = 231	3	58
-Dermal	Dermal NOAEL 24.40		See foot note[c]	
-Inhalation	1.38		8	167
Chronic Dietary	1.10		10	210
Fomesafen – Female Mouse				
S/I -Incidental Oral	0.50	NOAEL = 16 LOAEL = 176	32	352
-Dermal	100		See foot note[d]	
-Inhalation	0.50		32	352
Chronic Dietary	0.25		64	704
Imazosulfuron – Female Rat				
S/I -Incidental Oral	235.0	NOAEL = 222 LOAEL = 710	1.0	3.0
-Dermal	Assessment not required		Not Applicable	
-Inhalation	235.0		1.0	3.0
Chronic Dietary	75.0		3	9
Novaluron – Female Mouse				
S/I -Incidental Oral	4.38	NOAEL = 200 LOAEL = 2115	46	483
-Dermal	4.38		46	483
-Inhalation	4.38		46	483
Chronic Dietary	1.1		182	165
Penthiopyrad – Male Mouse				
S/I -Incidental Oral	27.0	NOAEL = 301 LOAEL = 1136	11	
-Dermal	Assessment not required		Not Applicable	
-Inhalation	27.0		11	
Chronic Dietary	27.0		11	
Pyraflufen-ethyl – Male / Female Rat				
S/I -Incidental Oral	20	NOAEL	M: 236 / F: 1114	M:12 / F:56 / M:47 / F:NE
-Dermal	Assessment not required		Not Applicable	
-Inhalation	20	LOAEL	M: 946 / F: NE	M:12 / F:56 / M:47 / F:NE
Chronic Dietary	20		M: 12 / F:56 / M:47 / F: NE	

Table 6. Impact of Immunotoxicity On Existing Risk Assessment

Exposure Scenario	Points of Departure (POD) (mg/kg/day)	Immunotoxicity NOAEL/LOAEL (mg/kg/day)	Magnitude Higher (x-fold) than POD to	
			NOAEL	LOAEL
Thiram – Male Mouse				
S/I -Incidental Oral	1.40	NOAEL = 25 LOAEL = 74	18	53
-Dermal	1.40		18	53
-Inhalation	1.40		18	53
Chronic Dietary	1.50		17	53
Trichlorfon – Female Rat				
S/I -Incidental Oral	Not Selected	NOAEL = 32 LOAEL = 164	Not Applicable	
-Dermal	Dermal NOAEL 100		See foot note[e]	
-Inhalation	3.45		9	48
Chronic Dietary	0.2		160	820
Triflumizol [e] – Female Mouse				
S/I -Incidental Oral	3.5	NOAEL = 29 LOAEL = 286	8	82
-Dermal	3.5		8	82
-Inhalation	3.5		8	82
Chronic Dietary	1.2[f]		24	238

NE = Not Established

a: *Buprofezin:* A route-specific dermal POD of 300 mg/kg/day was used for this scenario. The TDAR NOAEL (343 mg/kg/day in males and 79 mg/kg/day in females) with a 10% Dermal Absorption Factor (DAF) yielded a Dermal Equivalent Dose (DED) of 3430 mg/kg/day in males and 790 mg/kg/day in females. These values are 10-fold higher than the dermal POD used for this risk assessment. Therefore, the immunotoxicity study would not have been selected for the dermal scenario.

b: *Buprofezin:* The value (4.3 mg/kg/day) is the NOAEL (13 mg/kg/day) ÷ $UF_{Data\ Base}$ (3x).

c : *Fluazinam:* A route-specific dermal POD of 24 mg/kg/day was used for this risk assessment. The NOAEL of 11 mg/kg/day with a 4.5% DAF yielded a DED of 244 mg/kg/day which is 10-fold higher than the dermal POD used for this risk assessment. Use of the TDAR LOAEL (231 mg/kg/day) would have resulted in a DED of 513 mg/kg/day. Therefore, the immunotoxicity study would not have been selected for the dermal scenario.

d: *Fomesafen:* The lower NOAEL (16 mg/kg/day) in the immunotoxicity study is the result of large dose spacing (l0-fold); the LOAEL is 176 mg/kg/day . Therefore, the true NOAEL could have been higher and closer to the POD used for this risk assessment. However, the PoD is still protective of any potential immunotoxicity.

e: *Trichlorfon:* A route-specific dermal POD of 100 mg/kg/day was used for this risk assessment. Use of the TDAR NOAEL of 32 mg/kg/day with a 36% DAF yielded a DED of 89 mg/kg/day which is numerically equivalent to the dermal POD used for this risk assessment. The TDAR LOAEL of 164 mg/kg/day yields a DED of 456 mg/kg/day. The dermal POD is based on inhibition of cholinesterase activity (the most sensitive endpoint) seen following dermal exposures and is more appropriate for assessing dermal risk for this class of chemical (organophosphate) than the immunotoxicity endpoint.

f: *Triflumizole:* The extrapolated value (1.2 mg/kg/day) is the TDAR LOAEL (3.5 mg/kg/day) ÷ by the 3X Uncertainly Factor for the use of a LOAEL (UF_{LOAEL}).

IV. DISCUSSION

In 2007 the 40 CFR Part 158 Toxicology Data requirements were revised to include an immunotoxicity study (870.7800). This study provides an evaluation of the function of immune systems components (*i.e.*, humoral or cell-mediated type immunity) which allow a better characterization of potential immunotoxicity for a test chemical. The Agency, so far, has received 176 studies conducted for 160 chemicals; of those, 5 studies on 5 chemicals were classified as unacceptable due to various technical deficiencies in the conduct of these studies. Therefore, this retrospective analysis includes 171 studies conducted on 155 chemicals covering a wide variety of pesticide products and chemical classes.

All 155 chemicals were assessed by the TDAR using the PFCA and/or ELISA methods. In addition, a NK cell activity assay was conducted for 11 of these chemicals. The TDAR studies were conducted in either male or female mice or rats and for 14 chemicals the TDAR assays were conducted on both species. A number of different strains/stocks of mice and rats were tested.

For the majority of chemicals included in this analysis, there was a clear lack of toxicologically relevant effects on the functional immune system function. Out of 155 chemicals tested, 140 were reported to cause no effects on the TDAR up to the highest dose-tested (*i.e.* no evidence of immunotoxicity observed).

There were 15 chemicals (10%) that exhibited suppression of anti-SRBC IgM response at relatively high doses. For 9 of these chemicals, immunotoxicity was seen at the same dose or at higher doses at which systemic toxicity occurred in the study. For the other 6 chemicals, TDAR was seen in the absence of systemic toxicity within the immunotoxicity study. However, for risk assessment purposes, immunotoxicity was not the most sensitive endpoint in the toxicity data base for any of these chemicals. Consequently, the immunotoxicity studies would not have been used for risk assessment for these chemicals.

The Agency recommended using the most sensitive species (based on data from the existing Part 158 or other available toxicity data) for the immunotoxicity study with the mouse as the default species of choice. In this data set, more studies were conducted in rats (54%) than mouse (37%) and only 14 chemicals (9%) were tested in both species. Therefore, a definitive conclusion on the species sensitivity cannot be made at this time due to the low sample size (9%) of studies conducted in both species.

This data set included more studies conducted in females (mice, 69% and rats, 41%) than males (mice, 16% and rats, 31%). The higher representation of females for which immunotoxicity was seen is consistent with the Agency's guidance on sex selection which indicated that the female is the default gender of choice in the absence of any difference in sensitivity between sexes revealed by existing toxicity studies.

The conduct of the TDAR assay in the available studies is considered adequate to determine the effect of the test substance on the splenic anti-SRBC PFC response or serum anti-SRBC IgM levels, respectively. While the TDAR assay is designed to assess functional competency of the immune system, it is also important to note that structural immune parameters are routinely evaluated in other standard toxicity studies required for pesticide registration. Thus guideline studies provide acceptable and useful chemical information on the toxic potential, including potential effects on the immune system. More specifically, immune-related endpoints are evaluated by clinical pathology and histopathology in the subchronic (mice, rats and dogs) chronic (dog), reproduction (rat) and the chronic toxicity/carcinogenicity (mice and rats) studies.

For 125 of the 155 chemicals (81%) evaluated in this data set, there were no indicators of potential immunotoxicity in the standard repeated dose studies. Immune system endpoints seen in the remaining 30 chemicals (19%) included the following: alterations in hematology parameters; changes in spleen weights; and/or histopathological changes of the spleen. More appropriate indicators of immunotoxicity associated with hematology include changes in absolute or differential WBC count such as lymphocytosis or lymphopenia. However, these changes were not seen in the database. Therefore, the effects on the particular hematological parameters observed in the studies were not specific indicators of immunotoxic potential and are not reliable indicator of immunotoxicity in these studies.

For the 15 chemicals with a positive TDAR, 4 showed effects on immune system-related endpoints in the standard repeated dose studies. However, a closer examination of the parameters revealed that the observed effects in these standard studies were not attributable to immunotoxicity, but rather to other factors such as erythrocyte toxicity (*e.g.*, anemia), age of the test species (*e.g.*, seen at 78 weeks) or high dose exposure (*i.e.*, Limit Dose).

For 26 of the 140 chemicals (19%) that did not show an effect on TDAR, changes were seen in hematology parameters, spleen weights, and/or histopathological changes. Hematological changes (*i.e.*, HGB, HCT, MCV, MCHC, Heinz body and methemoglobin formation) are consistent with anemia and/or toxicity to the erythropoietic system rather than the immune system. In three cases, spleen weights were decreased or increased without any concomitant histopathological changes. Changes in absolute/relative organ weights are more specific to immune toxicity when they are observed in more than one lymphoid organ and associated with corroborative histopathology findings. The histopathological lesions of the spleen were limited to hemosiderosis and extramedullary hematopoiesis. Conditions associated with increased extramedullary hematopoiesis include anemia/ hemorrhage/hemolysis, systemic inflammation, splenic trauma/necrosis, decreased hematopoiesis by the bone marrow, and neoplasia. Consequently, in this group of studies effects seen in the spleen were not specific indicators of immunotoxicity. Therefore, the effects on the particular hematological parameters observed in the studies were not specific indicators of immunotoxic potential and are not reliable indicators of immunotoxicity in these studies.

To assess the impact of the immunotoxicity study on human health risk assessments, a comparison was made for the ratio of the NOAEL/LOAEL established for the TDAR to the PODs selected for different risk assessment scenarios. The NOAELs established in the immunotoxicity studies did not impact the PODs and toxicity endpoints of concern used for human health risk assessments. Consequently, immunotoxicity was not the most sensitive endpoint in the toxicity data base of the chemicals in this analysis. The Agency's analysis corroborates the findings and the conclusions reported by CLA.

V. CONCLUSIONS

The Agency recognizes that a science-based approach to testing that utilizes the best available knowledge on the chemical (physical chemical properties, hazard, pharmacokinetics, mechanistic data, structure activity relationships, etc.) should be used to determine: (1) whether a standard guideline study is needed or whether an enhanced guideline study or an alternative study should be conducted to assess potential hazard, or (2) in some cases to support a waiver for testing. Consistent with the Agency's commitment to the 3Rs (Replacement, Refinement and Reduction) of animal testing, the OPP is also committed to investigate approaches to reduce the number of animals needed for a thorough safety assessment of the chemicals of interest. Thus, OPP will be receptive to requests that it grant waivers for the immunotoxicity study. Such requests should be made on a case-by-case basis and include a scientifically sound, chemical-specific rationale. Also, the Agency will be receptive to approaches that effectively incorporate special immunotoxicity endpoints into the battery of routine toxicology studies if it would reduce animal usage while still providing the necessary information within the context of other toxicological endpoints. The following path to addressing the immunotoxicity data requirement is proposed:

1. Waiver Consideration

For pesticides that have not been evaluated by the TDAR study and for which a registrant requests a waiver, the Agency encourages registrants to provide a weight of evidence (WOE) based rationale. This WOE evaluation may include a variety of relevant scientific factors such as chemical structure, toxicological profile, primary target organ system(s), and mode of toxic action, as well as relevant information on structural analogs regarding immunotoxicity potential of the chemical class. Results from EPA's 2012 immunotoxicology study retrospective analysis may be useful as part of the WOE rationale for certain chemical classes. The Agency will review waiver requests and make case-by-case decisions.

2. **Alternative Ways to Sastisfying the Immunotoxicity Study Requirement:** An applicant or registrant may meet the requirement for immunotoxicity data by:

- Integrating immunotoxicity measures into any of several existing part 158 studies (*e.g.*, 28-day range finding or 90-day subchronic study);
- Conducting an extended One-Generation Reproduction Toxicity study (EOGRTS) *in lieu* of the TDAR guideline study. The immunotoxicity cohort of the EOGRTS (OECD Test Guideline 443) will satisfy the part 158 data requirement.
- Studies designed based on existing information and a hypothesized basis of the mode of immunotoxicity action of the chemical may be acceptable.

Registrants should consult the Agency for guidance / protocol concurrence for alternative studies.

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