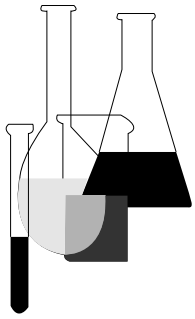
United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–96–351
June 1996

Health Effects Test Guidelines

OPPTS 870.7800
Immunotoxicity



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202–512–1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202–512–0132 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines.’’

870.7800 Immunotoxicity.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136 *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** This is a new guideline developed in the Office of Pesticide Programs.

(b) **Purpose.** The proposed tests are intended to provide information on suppression of the immune system which might occur as a result of repeated exposure to a test chemical, after dosing by either oral or parenteral routes. The selected studies provide quantitative data on the effects of a chemical pesticide on the numbers of cells in major lymphocyte subpopulations, and the functional responsiveness of major components of the immune system. While some information on potential immunotoxic effects may be obtained from hematology, lymphoid organ weights and histopathology, usually done as part of routine toxicity testing, there are data which demonstrate that these endpoints alone are not sufficient to predict immunotoxicity (see paragraphs (j)(7) and (j)(8) of this guideline). Therefore, the tests proposed here are intended to be used along with data from routine toxicity testing, to provide more accurate information on risk to the immune system. These tests were chosen, not only because they have been shown to predict accurately the immunotoxicity of a number of chemicals, but because they are readily reproducible from laboratory to laboratory and can be easily incorporated into routine toxicity testing. They do not represent a comprehensive assessment of immune functions.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

Antibodies or immunoglobulins (Ig) are part of a large family of glycoprotein molecules. They are produced by B cells in response to antigens, and bind specifically to the eliciting antigen. The different classes of immunoglobulins involved in immunity are IgG, IgA, IgM, IgD, and IgE. Antibodies are found in extracellular fluids, such as serum, saliva, milk, and lymph. Most antibody responses are T cell-dependent, that is, functional T and B lymphocytes, as well as antigen-presenting cells (usually macrophages), are required for the production of antibodies.

Cluster of differentiation (CD), refers to molecules expressed on the cell surface. These molecules are useful as distinct CD molecules are found on different populations of cells of the immune system. Antibodies against these cell surface markers, which are numbered (e.g. CD-4, CD-8), are used to identify and quantitate different cell populations.

Immunotoxicity refers to the ability of a test substance to suppress immune responses that could enhance the risk of infectious or neoplastic

disease, or to induce inappropriate stimulation of the immune system, thus contributing to allergic or autoimmune disease. These guidelines only address potential immune suppression.

Natural Killer (NK) cells are large granular lymphocytes which nonspecifically lyse cells bearing tumor or viral antigens. NK cells are *up*-regulated soon after infection by certain microorganisms, and are thought to represent the first line of defense against viruses and tumors.

T and *B cells* are subpopulations of lymphocytes which are activated in response to specific antigens (foreign substances, usually proteins). B cells produce antigen-specific antibodies, and a subpopulation of T cells is frequently needed to provide help for the antibody response. Other types of T cell participate in the direct destruction of cells expressing specific foreign (tumor or infectious agent) antigens on the cell surface.

(d) **Principle of the test methods**. (1) Rats and/or mice are exposed to the test and control substances for at least 30 days. They are immunized by intravenous injection of sheep red blood cells (SRBC) approximately 4 days (depending on the strain of animal) prior to the end of the exposure. At the end of the exposure period, the anti-SRBC plaque-forming cell (PFC) assay or enzyme-linked immunosorbent assay (ELISA) is performed to determine the effects of the test substance on either splenic IgM PFC response, or serum IgM levels, respectively.

(2) Expression of phenotypic markers for major lymphocyte populations (total T (CD3), total B (CD-45R), NK (using a marker specific to the species and strain of animal used), and T subpopulations (CD4 and CD8)), as assessed by flow cytometry, is used to determine the effects of the test substance on either splenic or peripheral-blood lymphocyte populations. A functional test for NK cells may be substituted for flow cytometric analysis. For tests performed using cells or sera from blood (ELISA or phenotypic markers), it is not necessary to destroy the animals, since immunization with SRBC's at 30 days is not expected to affect the results of other assays included in subchronic or longer-term studies markedly (see paragraph (j)(6) of this guideline). Hence, these assays could be incorporated into longer-term studies, when these studies are required.

(e) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects or if toxic effects would not be expected based upon data of structurally related compounds, a full study using three dose levels might not be necessary.

(f) **Test procedures**—(1) **Animal selection**—(i) **Species and strain**. These tests are intended for use in the mouse and rat. Commonly used laboratory strains should be employed. All test animals should be free of pathogens and internal and external parasites. Females should be

nulliparous and nonpregnant. The species, strain, and source of the animals must be identified.

(ii) **Age.** (A) Young, healthy animals should be employed. At the commencement of the study, the weight variation of the animals used should not exceed ± 20 percent of the mean weight.

(B) Dosing should begin when the test animals are between 6 and 8 weeks old.

(iii) **Sex.** Equal numbers of animals of each sex should be used at each dose level, and the females should be nulliparous and nonpregnant.

(iv) **Numbers.** (A) At least 10 animals should be included in each dose and control group for the anti-SRBC PFC assay or ELISA, and 6 animals per group for the phenotypic analysis of immune cell populations.

(B) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required.

(C) Each animal must be assigned a unique identification number. Dead animals, their preserved organs and tissues, and microscopic slides should be identified by reference to the animal's unique number.

(v) **Husbandry.** (A) Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g. morbidity, excitability) may indicate a need for individual caging.

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C.

(C) The relative humidity of the experimental animal rooms should be between 30 and 70 percent.

(D) Where lighting is artificial, the sequence should be 12 h light/ 12 h dark.

(E) Control and test animals should be maintained on the same type of bedding and receive feed from the same lot. The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. Rodents should be fed and watered ad libitum with food replaced at least weekly.

(F) The study should not be initiated until the animals have been allowed a period of acclimatization/quarantine of at least 1 week to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine.

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or dilu-

ent is needed, it should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance. It is recommended that an aqueous solution be used wherever possible. A solution in oil may be used if solubility is a problem. Other vehicles may be considered, but only as a last resort.

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound and, if technically feasible, the name and quantities of any known contaminants and impurities.

(iii) If the test or positive control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study. Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study. Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(3) **Control groups.** (i) A concurrent, vehicle-treated control group is required.

(ii) A separate untreated control group is required if the toxicity of the vehicle is unknown.

(iii) A positive control group with a known immunosuppressant (e.g. cyclophosphamide) is useful in the interpretation of the results or verification of the assay sensitivity, and should be included in the study. When used, a group of at least five animals should be given the immunosuppressive chemical.

(4) **Dose levels.** (i) In repeated-dose toxicity tests, unless a limit test using 1,000 mg/kg body weight is performed as specified in paragraph (e) of this guideline, it is desirable to have a dose-response relationship and a no observed immunotoxic effect level. Therefore, at least three dose levels and a negative control should be used.

(ii) The highest dose level should not produce significant stress, malnutrition, or fatalities, but should produce some measurable sign of general toxicity (e.g. 10 percent loss of body weight).

(iii) The lowest dose level should not produce any evidence of immunotoxicity.

(5) **Administration of the test substance.** (i) The test substance, vehicle, or positive control substance is administered for 30 days, usually

by the oral route, for the anti-SRBC PFC or ELISA assay. Because there is fairly rapid turnover of many of the cells in the immune system, 30 days is considered sufficient for screening purposes. This shorter time period is in deference to the fact that a group of animals may have to be specifically dedicated to these tests. A dedicated group of animals is not required for flow cytometric analysis. Under ordinary circumstances this test should be done after 90 days of administration; however, if phenotypic analysis is performed in conjunction with a repeated dose dermal toxicity study, a shorter administration period may be allowed.

(ii) If the test substance is administered by gavage, the animals are dosed with the test substance on a 7-days-per-week basis. However, based primarily on practical considerations, dosing by gavage on a 5-days-per-week is acceptable. If the test substance is administered in the drinking water, or mixed directly into the diet, exposure should be on a 7-days-per-week basis.

(A) All animals should be dosed by the same method during the entire experimental period.

(B) For substances of low toxicity, it is important to ensure that when administered in the diet, the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet, either a constant dietary concentration (parts per million) or a constant dose level in terms of the animal's body weight should be used; the alternative used should be specified.

(C) For a substance administered by gavage, the dose should be given at approximately the same time each day, and adjusted at intervals (weekly for mice, twice per week for rats) to maintain a constant dose level in terms of the animal's body weight.

(6) **Observation period.** Duration of the observation period should be 30 days for the PFC or ELISA determinations, and 90 days for phenotypic analysis.

(7) **Observation of animals.** (i) Observations should be made at least once each day for morbidity and mortality. Appropriate actions should be taken to minimize loss of animals to the study (e.g. necropsy of those animals found dead and isolation or euthanasia of weak or moribund animals).

(ii) Careful clinical examination should be made at least once a week. Observations should be detailed and carefully recorded, preferably using explicitly defined scales. Observations should include, but not be limited to: evaluation of skin and fur, eyes and mucous membranes; respiratory and circulatory effects; autonomic effects, such as salivation; central nervous system effects, including tremors and convulsions, changes in the level of motor activity, gait and posture, reactivity to handling or sensory stim-

uli, grip strength, and stereotypes or bizarre behavior (e.g. self-mutilation, walking backwards).

(iii) Signs of toxicity should be recorded as they are observed including the time of onset, degree and duration.

(iv) Food and water consumption should be determined weekly.

(v) Animals should be weighed just prior to dosing, weekly (twice per week for rats) thereafter, and just prior to euthanasia.

(vi) Any moribund animals should be removed and euthanized when first noticed. Necropsies should be conducted on all moribund animals, and on all animals that die during the study.

(g) **Immunotoxicity tests.** The studies required to screen the potential of a test substance to affect the immune system are:

(1) **Functional tests.** Either a PFC assay or an ELISA may be used to determine the response to antigen administration.

(i) **Antibody plaque-forming cell (PFC) assay**. The Jerne and Nordin antibody plaque-forming cell assay, as modified under paragraph (j)(1) of this guideline or as described in detail (see paragraph (j)(2) of this guideline), is used to demonstrate the effects of exposure (30 days) to a test substance on antibody-producing cells from the spleen. The following points should be considered when conducting this assay:

(A) The T cell-dependent antigen, sheep red blood cells (SRBC), should be injected intravenously, usually at 26 days after the first dosing with the test substance. Although the optimum response time is usually 4 days after immunization, some strains of test animal may deviate from this time point. Hence, the strain to be used should be evaluated for the optimum day for PFC formation after immunization.

(B) The activity of each new batch of complement should be determined. For any given study, the SRBCs should be from a single sheep, or pool of sheep, for which the shelf life and dose for optimum response has been determined.

(C) Modifications of the above-cited PFC assay exist (for example see paragraph (j)(4) or paragraph (j)(9) of this guideline) and may prove useful; however, the complete citation should be made for the method used, any modifications to the method should be reported, and the source and, where appropriate, the activity or purity of important reagents should be given. Justification or rationale is to be provided for each protocol modification.

(D) It is recommended that samples be randomized and coded for PFC analysis, so that the analyst is unaware of the treatment group of each sample examined.

(E) Spleen cell viability is to be determined.

(F) The numbers of IgM PFC per spleen, and the number of IgM PFC per 10^6 spleen cells must be reported.

(ii) **Immunoglobulin quantification: Enzyme-linked immunosorbent assay (ELISA)**. As an alternative to a PFC assay, the effects of the test substance on the antibody response to antigen may be determined by an ELISA (see paragraphs (j)(9) and (j)(5) of this guideline for a comparison between the PFC and ELISA assays for immunotoxicity assessment). Test animals are immunized with SRBCs as for the PFC assay. IgM titers in the serum of each test animal are determined (usually 4 days after immunization). As with the PFC assay, the optimum dose of SRBCs and optimum time for collection of the sera need to be determined for the species and strain of animal to be tested. Detailed methods are described under paragraph (j)(10) of this guideline).

(2) **Enumeration of splenic or peripheral blood T cells, B cells, and NK cells.** The phenotypic analysis of T cell, B cell, and NK cell populations from the spleen or peripheral blood by flow cytometry should be performed after ninety days of dosing; this may be done in conjunction with ninety-day (oral, dermal, and inhalation) toxicity studies, carcinogenicity studies, and chronic toxicity studies, when these studies are required. Methods are described under see paragraphs (j)(4) and (j)(3) of this guideline.

(h) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing effects, the types of effects and the percentage of animals displaying each type of effect.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods including significance criteria should be selected during the design of the study.

(2) **Evaluation of study results.** The findings of an immunotoxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of other toxic effects. The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted test should provide a satisfac-

tory estimation of a no-observed-effect level. It also can indicate the need for an additional study and provide information on the selection of dose levels.

(3) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, 40 CFR part 160, and the OECD principles of GLP (ISBN 92–64–12367–9), the following specific information should be reported:

(i) The test substance characterization should include:

(A) Chemical identification.

(B) Lot or batch number.

(C) Physical properties.

(D) Purity/impurities.

(E) Identification and composition of any vehicle used.

(ii) The test system should contain data on:

(A) Species, strain, and rationale for selection of animal species, if other than that recommended

(B) Age, body weight data, and sex.

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods.

(D) Identification of animal diet.

(iii) The test procedure should include the following data:

(A) Method of randomization used.

(B) Full description of experimental design and procedure.

(C) Dose regimen including levels, methods, and volume.

(4) **Test results**. (i) Group animal data: Tabulation of toxic response data by species, strain, sex and exposure level for:

(A) Number of animals exposed.

(B) Number of animals showing signs of toxicity.

(C) Number of animals dying.

(ii) Individual animal data. Data should be presented as summary (group mean) as well as for individual animals.

(A) Date of death during the study or whether animals survived to termination.

(B) Date of observation of each abnormal sign and its subsequent course.

(C) Body weight data.

(D) Feed and water consumption data, when collected.

(E) Results of immunotoxicity screen.

(F) Necropsy findings of animals that were found moribund and euthanized or died during the study.

(G) Statistical treatment of results, where appropriate.

(i) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment. The study must be conducted in compliance with the Good Laboratory Practice (GLP) regulations as described by the Agency (40 CFR parts 160 and 792) and the OECD principles of GLP (ISBN 92–64–12367–9).

(j) **References.** The following are publications that either provide useful protocols for the design of immunotoxicity studies, or contain citations for useful protocols.

(1) Cunningham, A.J. A method of increased sensitivity for detecting single antibody-forming cells. *Nature* 207:1106–1107 (1965).

(2) Holsapple, M.P. The plaque-forming cell (PFC) response in Immunotoxicology: An approach to monitoring the primary effector function of B lymphocytes. In *Methods in Immunotoxicology* (G.R. Burleson, J.H. Dean, and A.E. Munson, Eds.), Vol. 1, pp. 71–108. Wiley-Liss, New York (1995).

(3) Cornacoff, J.B. et al. Phenotypic identification of peripheral blood mononuclear leukocytes by flow cytometry as an adjunct to immunotoxicity evaluation. In *Methods in Immunotoxicology* (G.R. Burleson, J.H. Dean, and A.E. Munson, Eds.), Vol. 1, pp 211–226, Wiley-Liss, New York (1995).

(4) Ladics, G.S. and Loveless, S.E. Cell surface marker analysis of splenic lymphocyte populations of the CD rat for use in immunotoxicological studies. *Toxicology Methods* 4: 77–91 (1994).

(5) Ladics, G.S. et al. Evaluation of the humoral immune response of CD rats following a 2-week exposure to the pesticide carbaryl by the oral, dermal, or inhalation routes. *Journal of Toxicology and Environmental Health* 42:143–156 (1994).

(6) Ladics. et al. Possible incorporation of an immunotoxicological functional assay for assessing humoral immunity for hazard identification purposes in rats on standard toxicology study. *Toxicology* 96:225–238 (1995).

(7) Luster, M.I. et al. Risk assessment in immunotoxicology I. Sensitivity and predictability of immune tests. *Fundamentals of Applied Toxicology* 18:200–210 (1992).

(8) Luster, M.I. et al. Risk Assessment in Immunotoxicology II. Relationships Between Immune and Host Resistance Tests. *Fundamentals of Applied Toxicology* 21:71–82 (1993).

(9) Temple, L. et al. Comparison of ELISA and plaque-forming cell assays for measuring the humoral immune response to SRBC in rats and mice treated with benzo[a]pyrene or cyclophosphamide. *Fundamentals of Applied Toxicology* 21:412–419 (1993).

(10) Temple, L. et al. In *Methods in Immunotoxicology* (G.R. Burleson, J.H. Dean, and A.E. Munson, Eds.), Vol. 1, pp 137–157, Wiley-Liss, New York (1995).