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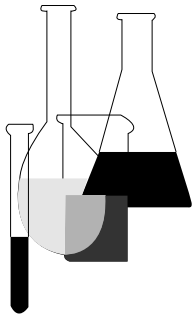
Biochemicals Test Guidelines

OPPTS 880.3550
Immunotoxicity



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.*).

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OPPTS 880.3550 Immunotoxicity.

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

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is OPP guideline 152–18.

(b) **Immunotoxicity studies with biochemical pest control agents (BPCAs): Tier I**—(1) **When required.** Data on alterations of immune responses are conditionally required to support the registration of each manufacturing-use product and each end-use product. These studies will be required when there is a requirement for subchronic (90-day) oral OPPTS 870.3100, dermal OPPTS 870.3250, or inhalation OPPTS 870.3465 studies. The routes of exposure for the immunotoxicity studies will be analogous to the route of exposure for each required subchronic study.

(2) **Purpose.** Immunotoxicity data provide information on health hazards likely to arise from subchronic exposure to a test chemical, usually after dosing by the oral route. Tests are selected to provide qualitative and quantitative data on the capacity of a chemical to adversely affect components of antibody-mediated and specific and non-specific cell-mediated immunity.

(3) **Definitions.** ''*Immunotoxicity*'' refers to the ability of a test substance to induce dysfunction or inappropriate suppressive or stimulatory responses in components of the immune system.

(4) **Principles of the test methods.** The test methods are designed to provide information on the ability of a test substance to alter or impair various components of the immune system. Parameters that are evaluated include immune system tissue and organ weights and cellularity, clinical blood chemistry, hematology, humoral immunity, and cellular immunity. The test substance is administered in graduated doses to several groups of experimental animals, one dose level per group, for a period of at least 30 days. Animals are observed daily to detect any signs of clinical toxicity. At the end of the dosing period, animals are sacrificed and parameters of the immune system are examined. In some tests, sensitization of the animals with an appropriate immunogen is required.

(5) **Substance to be tested.** The technical grade of each active ingredient should be tested.

(c) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** The mouse and rat are the preferred species. Commonly used laboratory strains should be employed. If another species is used, then justification or rationale should be provided for the selection. All test animals should

be free of parasites and pathogens. Females should be nulliparous and non-pregnant.

(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% of the mean weight.

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

(iii) **Sex.** A single sex of test animal may be used in the Tier I studies. When there is reason to believe that one sex may be more sensitive to effects of the test substance, then that sex must be used.

(iv) **Numbers.** (A) At least 10 animals should be included in each dose group and in each control group for each immunological parameter evaluated.

(B) [Reserved]

(2) **Control groups.** (i) A concurrent vehicle-treated control group is required, and should contain a sufficient number of additional test animals to serve as controls for studies that may be needed to evaluate the reversibility, persistence, or delayed occurrence of immunotoxic effects (see OPPTS 880.3800 (d)(1)).

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

(iii) A positive control group of at least 5 animals per assay, dosed with a known immunosuppressant, is required (where indicated below) to verify assay sensitivity.

(3) **Satellite group.** A satellite control group of at least 20 animals, treated with the test substance at the high dose for 30 days, is suggested for possible observation of reversibility, persistence, or delayed occurrence of immunotoxic effects after appropriate post-treatment time periods (see OPPTS 880.3800 (d)(1)).

(4) **Dose levels.** (i) In subchronic toxicity tests, it is desirable to have a dose-response relationship and also a no observed immunotoxic effect level. Therefore, at least three dose levels and a control should be used.

(ii) The highest dose level should not produce significant stress, malnutrition, or fatalities, but ideally should produce some measurable sign of general toxicity.

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

(5) **Administration of the test substance.** The test substance or vehicle is administered for at least 30 days by oral exposure, and/or by dermal exposure, and/or by inhalation exposure in a manner analogous to the respective required 90-day studies.

(6) **Observation period.** (i) Duration of observation should be for at least 30 days.

(ii) Animals in the satellite group, if needed for scheduled follow-up examinations, should be kept for a further 30 days without treatment to detect recovery from or persistence of immunotoxic effects, or for the detection of delayed occurrence of immunotoxic effects.

(7) **Observation of animals.** (i) A careful cageside examination should be made on each animal at least once each day. Clinical signs of toxicity should be recorded as they are observed, including the time of onset, degree and duration. Cageside observations should include, but not be limited to, changes in:

(A) Skin and fur.

(B) Eyes and mucous membranes.

(C) Respiratory system.

(D) Autonomic and central nervous system.

(E) Circulatory system.

(F) Somatomotor activity.

(G) Behavior pattern.

(H) Resistance to infection.

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

(iii) Animals should be weighed just prior to dosing, weekly thereafter, and just prior to sacrifice.

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

(8) **Clinical examinations.** The following examinations should be made on 10 animals per dose group and 10 control group animals at the end of the test period. These animals should be fasted overnight, prior to sacrifice.

(i) Hematology determinations which are considered to be appropriate are:

(A) Hematocrit.

(B) Hemoglobin concentration.

(C) Erythrocyte count.

(D) Total and differential leucocyte count.

(E) Platelet count.

(ii) Clinical biochemistry determinations on blood which are considered to be appropriate are:

(A) Glucose.

(B) Serum glutamic-pyruvic transaminase.

(C) Urea nitrogen.

(D) Albumin.

(E) Total serum protein measurements.

(F) Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(9) **Gross necropsy.** (i) All animals should be subjected to a limited gross necropsy, which should include:

(A) Body weight determination.

(B) Wet weight determinations of the thymus and spleen, as soon as possible after dissection to avoid drying.

(ii) [Reserved]

(10) **Tissue preparation.** (i) The following organs and tissues, or representative samples thereof, from 10 animals in each dose group and 10 control group animals should be preserved in a suitable medium for possible histopathological examination:

(A) Thymus.

(B) Spleen.

(C) Liver.

(D) Lungs.

(E) Kidneys.

(F) Bone marrow (either femur, sternum, or rib at the costochondrial junction).

(G) Representative lymph nodes (mucosa-associated and peripheral).

(H) Adrenals.

(I) Pituitary.

(J) Ovaries or testes.

(K) All gross lesions.

(ii) [Reserved]

(11) The cellularity and cell viability of the spleen, thymus, and bone marrow should be determined for at least 10 animals per dose group and 10 control group animals.

(12) Conventional histopathology should be performed on all gross lesions.

(13) Histopathology should be done on the above tissues/organs when indicated by the observation of adverse immunological effects in the specific and non-specific cell-mediated immune response sections of this study.

(d) **Immunotoxicity tests.** Components of the immune system, and the studies suggested to screen the potential of a test substance to affect them, are:

(1) **Humoral immunity.** Either an antibody plaque-forming cell assay or a determination of immunoglobulin titers after antigen administration are required for assessment of the effect of a test substance on humoral immune responses.

(i) **Antibody plaque-forming cell (PFC) assay.** The Jerne and Nordin antibody plaque-forming cell assay, as modified by Cunningham and Cunningham and Szenberg (see paragraphs (g)(1) and (g)(2) of this guideline), is used to demonstrate the effects of subchronic exposure (30 days) to a test substance on antibody-producing cells enumerated from the spleen. The following points should beconsidered 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. Each species and strain of test animal should be evaluated for the optimum day for PFC formation after immunization.

(B) The activity of each new batch of complement should be determined.

(C) Modifications of the above-cited PFC assay exist (for example, see Temple, et al. in paragraph (f)(22) 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) A positive control group of test animals treated with a known immunosuppressant chemical (e.g., cyclophosphamide) is required to verify the sensitivity of the assay.

(E) It is recommended that animals be randomized at sacrifice, for PFC analysis.

(F) Spleen cell viability is to be determined.

(ii) **Immunoglobulin quantification.** Effects of the test substance on the antibody response to antigen are to be determined. Test animals are immunized with an appropriate thymus-dependent antigen, followed by a secondary challenge with antigen at an appropriate time. IgG and IgM titers in the serum of each test animal are determined. Immunoglobulin analyses should be done with sufficient frequency so that the primary and secondary antibody responses of treated and control animals can be adequately compared. At least 30 days of dosing with the test substance are required before the final IgG and IgM measurements are made. The following points should be considered when measuring antibody responses to antigen:

(A) The technique used for determining IgG and IgM titers should be sufficiently sensitive so that values from individual animals can be determined. The enzyme-linked immunosorbent assay (ELISA) is considered as a sensitive, reliable, and reproducible technique.

(B) [Reserved]

(2) **Specific cell-mediated immunity.** One of the following three assays are required for an assessment of subchronic (30 day) exposure to a test substance on specific cell-mediated immunity:

(i) **One-way mixed lymphocyte culture (MLC) assay.** A one-way mixed lymphocyte culture assay is used to demonstrate the effects of subchronic exposure (30 days) to a test substance on lymphocyte blastogenesis as stimulated by allogeneic lymphocytes. Lymphocyte blastogenesis is measured by incorporation of radiolabel (usually ^{3}H-thymidine) into DNA. The following points should be considered when conducting this assay:

(A) Unblocked ‘‘responder’’ cells typically are prepared under aseptic conditions from the spleens of control and treated animals.

(B) ‘‘Stimulator’’ cells are prepared under aseptic conditions from the spleens of an allogeneic, untreated species, and DNA synthesis is blocked by mitomycin C or X-irradiation.

(C) Viability of cells in ‘‘responder’’ and ‘‘stimulator’’ populations is determined.

(D) Assay controls should be included in triplicate or quadriplicate to account for the efficiency of the harvesting technique; to insure non-reactivity of stimulator cells; and to determine the baseline levels of DNA synthesis.

(E) Both naive and, if included, vehicle control groups are to be analyzed.

(F) The incorporation of radiolabel in each culture of responder cells is a measure of blastogenesis, and is expressed as CPM. Data are expressed as nCPM, which is the mean net CPM of replicate stimulator-responder combinations minus the mean net CPM incorporated due to baseline levels of DNA synthesis. The percent differences between treated and control animals is expressed as: [1-(nCPM treated animals/nCPM untreated animals)] $\times$ 100.

(G) Numerous variations in protocol exist for the one-way MLC assay, and thus, citation to the method used and details of procedures employed should be provided, along with the source of important reagents and materials, and, if appropriate, their purity.

(H) A positive control group of test animals treated with an appropriate known immunosuppressant is required to verify the sensitivity of the assay.

(ii) **Delayed-type hypersensitivity (DTH) reaction.** In vivo assays exist that are useful in demonstrating the effects of a test substance on an induced DTH reaction in test animals. In general, test animals are sensitized, and later challenged with an appropriate thymus-dependent antigen. At 24-48 hours after challenge, DTH reactions in treated animals are compared to DTH reactions in control group animals. The following points should be considered when performing an in vivo DTH reaction assay:

(A) A number of different DTH reaction assays exist; however, the particular assay selected should be demonstrated as sensitive, reproducible, and appropriate to the test animal species used.

(B) Parameters that vary among assays include the nature of the immunizing and challenge agents used; the number and route of immunizing injections; the time of challenge; and the use of isotope injection. These, and other related parameters, must be demonstrated to be appropriate for generating a sufficient DTH reaction in the test animal species used.

(C) Assays should be designed so that animals are treated with the test substance for at least 30 days prior to measuring DTH reactions.

(iii) **Cytotoxic T-lymphocyte (CTL) assay.** CTL assays are useful in demonstrating effects of subchronic (30 day) exposure to a test substance on the generation of cytotoxic T-lymphocytes. In this assay, an appropriate allogeneic tumor is used for CTL induction (induction can be accomplished either in vivo or in vitro). Splenocytes from treated and untreated test animals are then incubated with ^{51}Cr-labeled allogeneic tumor cells. The amount of radiolabel released from the target cells after incubation with the effector cells for four hours is used as a measure of T-lymphocyte cytolysis. The following points should be considered when performing a CTL assay:

(A) Assay controls should be included to account for spontaneous release of radiolabel from target cells in the absence of effector cells, and also for the determination of total release of radiolabel.

(B) It must be demonstrated that CTLs can be generated in the test animal selected for study, and the assay protocol used must be appropriate for CTL induction in the test animals.

(C) A number of different protocols exist that may prove useful. However, complete citation should be made to the method used, modifications should be reported, and, where appropriate, the source, activity, and/or purity of important reagents should be provided.

(3) **Non-specific cell-mediated immunity.** Assays that evaluate the function of natural killer cells and macrophage numbers and phagocytosis are required to determine the effects of subchronic (30 day) exposure to a test substance on non-specific, cell-mediated immunity.

(i) **Natural killer (NK) cell activity.** The microculture method of Reynolds and Herberman (see paragraph (f)(17) of this guideline) is recommended to demonstrate effects of subchronic (30 day) exposure to a test substance on spontaneous cytotoxic activity. In this assay, splenocytes from treated and untreated test animals are incubated with ^{51}Cr-labeled YAC-1 lymphoma cells. The amount of radiolabel released from the target cells after incubation with the effector cells for four hours is used as a measure of natural killer cytolysis. The following points should be considered when using the NK cell assay:

(A) Assay controls should be included to account for spontaneous release of radiolabel from target cells in the absence of effector cells, and also for the determination of total release of radiolabel.

(B) Target cells other than YAC-1 lymphoma cells may be appropriate for use, but, in all cases, target cell viability is to be determined.

(C) Modifications of the protocol exist that may prove useful. However, complete citation should be made to the method used. Modifications should be reported, and, where appropriate, the source, activity, and/or purity of important reagents should be given. Justification or rationale is to be provided for each protocol modification.

(ii) **Macrophages.** Assays should be performed at 30 days after dosing to evaluate effects of subchronic (30 day) exposure of test animals to a test substance on macrophage numbers and on macrophage phagocytosis. Assays to be done include:

(A) A total and differential count of resident peritoneal cell numbers.

(B) An evaluation of phagocytosis of particles (e.g., fluorescent latex beads) by peritoneal cells, in the presence and absence of augmentation factors (e.g., gamma interferon or bacterial lipopolysaccharide).

(C) A number of useful phagocytosis assays exist; thus, a description of and citation for the assay procedures used and a justification or rationale for any protocol modifications should be provided.

(e) Reporting. The following reporting requirements should be met in addition to the requirements set forth in § 150-4 of this subdivision:

(1) Statistical methods used to analyze data.

(2) Details on quarantine and housing of test animals.

(3) Historical control values for immune system parameters.

(4) Methods used to minimize variability in data from immune system parameter assays.

(f) **Tier progression.** (1) If dysfunction or impairment of the components of the immune system are indicated in any of the Tier I immunotoxicity tests, then the applicable Tier II immunotoxicity studies (OPPTS 880.3800) shall be required as specified in 40 CFR 158.165. Tier II immunotoxicity testing also may be required if the data from the Tier I tests cannot be definitively interpreted, or if data from other sources indicate that the test substance, or structurally related substances (including metabolites and degradation products), are immunotoxic.

(2) If dysfunction or impairment of the components of the immune system clearly are not indicated after testing at the Tier I level, or if no data are available, or become available, from other sources which indicate that the test substance, or structurally-related substances (including metabolites and degradation products) are immunotoxic, then no further testing is required.

(g) **References.** The following references should be consulted for additional background material on this test guideline. The following are pub-

lications that either provide useful protocols for the design of immunotoxicity studies, or contain citations for useful protocols.

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