Appendix I

Guidelines for Work with Toxins of Biological Origin

Biological toxins comprise a broad range of poisons, predominantly of natural origin but increasingly accessible by modern synthetic methods, which may cause death or severe incapacitation at relatively low exposure levels.[1,2] Laboratory safety principles are summarized herein for several toxins currently regulated as "Select Agent Toxins," including BoNT, SE, ricin and selected LMW toxins. Additional details are provided in the agent summary statements.

GENERAL CONSIDERATIONS FOR TOXIN USE

Laboratory work with most toxins, in amounts routinely employed in the biomedical sciences, can be performed safely with minimal risk to the worker and negligible risk to the surrounding community. Toxins do not replicate, are not infectious, and are difficult to transmit mechanically or manually from person to person. Many commonly employed toxins have very low volatility and, especially in the case of protein toxins, are relatively unstable in the environment; these characteristics further limit the spread of toxins.

Toxins can be handled using established general guidelines for toxic or highly-toxic chemicals with the incorporation of additional safety and security measures based upon a risk assessment for each specific laboratory operation.[3,4] The main laboratory risks are accidental exposure by direct contamination of mouth, eyes or other mucous membranes; by inadvertent aerosol generation; and by needle-sticks or other accidents that may compromise the normal barrier of the skin.

TRAINING AND LABORATORY PLANNING

Each laboratory worker must be trained in the theory and practice of the toxins to be used, with special emphasis on the nature of the practical hazards associated with laboratory operations. This includes how to handle transfers of liquids containing toxin, where to place waste solutions and contaminated materials or equipment, and how to decontaminate work areas after routine operations, as well as after accidental spills. The worker must be reliable and sufficiently adept at all required manipulations before being provided with toxin.

A risk assessment should be conducted to develop safe operating procedures before undertaking laboratory operations with toxins; suggested "pre-operational checklists" for working with toxins are available.[4] For complex operations, it is recommended that new workers undergo supervised practice runs in which the exact laboratory procedures to be undertaken are rehearsed without active toxin. If toxins and infectious agents are used together, then both must be considered when containment equipment is selected and safety procedures are developed. Likewise, animal safety practices must be considered for toxin work involving animals.

Each laboratory that uses toxins should develop a specific chemical hygiene plan. The National Research Council has provided a review of prudent laboratory practices when handling toxic and highly toxic chemicals, including the development of chemical hygiene plans and guidelines for compliance with regulations governing occupational safety and health, hazard communication, and environmental protection.[5]

An inventory control system should be in place to account for toxin use and disposition. If toxins are stored in the laboratory, containers should be sealed, labeled, and secured to ensure restricted access; refrigerators and other storage containers should be clearly labeled and provide contact information for trained, responsible laboratory staff.

Laboratory work with toxins should be done only in designated rooms with controlled access and at pre-determined bench areas. When toxins are in use, the room should be clearly posted: "Toxins in Use—Authorized Personnel Only." Unrelated and nonessential work should be restricted from areas where stock solutions of toxin or organisms producing toxin are used. Visitors or other untrained personnel granted laboratory access must be monitored and protected from inadvertently handling laboratory equipment used to manipulate the toxin or organism.

SAFETY EQUIPMENT AND CONTAINMENT

Routine operations with dilute toxin solutions are conducted under BSL-2 conditions with the aid of personal protective equipment and a well-maintained BSC or comparable engineering controls.[6] Engineering controls should be selected according to the risk assessment for each specific toxin operation. A certified BSC or chemical fume hood will suffice for routine operations with most protein toxins. Low molecular weight toxin solutions, or work involving volatile chemicals or radionucleotides combined with toxin solutions, may require the use of a charcoal-based hood filter in addition to HEPA filtration.

All work with toxins should be conducted within the operationally effective zone of the hood or BSC, and each user should verify the inward airflow before initiating work. When using an open-fronted fume hood or BSC, workers should wear suitable laboratory PPE to protect the hands and arms, such as laboratory coats, smocks, or coveralls and disposable gloves. When working with toxins that pose direct percutaneous hazards, special care must be taken to select gloves that are impervious to the toxin and the diluents or solvents employed. When conducting liquid transfers and other operations that pose a potential splash or droplet hazard in an open-fronted hood or BSC, safety glasses and disposable facemask, or a face shield, should be worn.

Toxin should be removed from the hood or BSC only after the exterior of the closed primary container has been decontaminated and placed in a clean secondary container. Toxin solutions, especially concentrated stock solutions, should be transported in leak/spill-proof secondary containers. The interior of the hood or BSC should be decontaminated periodically, for example, at the end of a series of related experiments.

Until thoroughly decontaminated, the hood or BSC should be posted to indicate that toxins remain in use, and access should remain restricted.

Selected operations with toxins may require modified BSL-3 practices and procedures**.** The determination to use BSL-3 is made in consultation with available safety staff and is based upon a risk assessment that considers the variables of each specific laboratory operation, especially the toxin under study, the physical state of the toxin (solution or dry form), the total amount of toxin used relative to the estimated human lethal dose, the volume of the material manipulated, the methodology, and any human or equipment performance limitations.

INADVERTENT TOXIN AEROSOLS

Emphasis must be placed on evaluating and modifying experimental procedures to eliminate the possibility of inadvertent generation of toxin aerosols. Pressurized tubes or other containers holding toxins should be opened in a BSC, chemical fume hood, or other ventilated enclosure. Operations that expose toxin solutions to vacuum or pressure, for example sterilization of toxin solutions by membrane filtration, should always be handled in this manner, and the operator should also use appropriate respiratory protection. If vacuum lines are used with toxin, they should be protected with a HEPA filter to prevent entry of toxins into the line.

Centrifugation of cultures or materials potentially containing toxins should only be performed using sealed, thick-walled tubes in safety centrifuge cups or sealed rotors. The outside surfaces of containers and rotors should be routinely cleaned before each use to prevent contamination that may generate an aerosol. After centrifugation, the entire rotor assembly is taken from the centrifuge to a BSC to open it and remove its tubes.

MECHANICAL INJURIES

Accidental needle-sticks or mechanical injury from “sharps” such as glass or metal implements pose a well-known risk to laboratory workers, and the consequences may be catastrophic for operations involving toxins in amounts that exceed a human lethal dose.

Only workers trained and experienced in handling animals should be permitted to conduct operations involving injection of toxin solutions using hollow-bore needles. Discarded needles/syringes and other sharps should be placed directly into properly labeled, puncture-resistant sharps containers, and decontaminated as soon as is practical.

Glassware should be replaced with plastic for handling toxin solutions wherever practical to minimize the risk of cuts or abrasions from contaminated surfaces. Thin-walled glass equipment should be completely avoided. Glass Pasteur pipettes are particularly dangerous for transferring toxin solutions and should be replaced with disposable plastic pipettes. Glass chromatography columns under pressure must be enclosed within a plastic water jacket or other secondary container.

ADDITIONAL PRECAUTIONS

Experiments should be planned to eliminate or minimize work with dry toxin (e.g. freeze-dried preparations). Unavoidable operations with dry toxin should only be undertaken with appropriate respiratory protection and engineering controls. Dry toxin can be manipulated using a Class III BSC, or with the use of secondary containment such as a disposable glove bag or glove box within a hood or Class II BSC. “Static-free” disposable gloves should be worn when working with dry forms of toxins that are subject to spread by electrostatic dispersal.

In specialized laboratories, the intentional, controlled generation of aerosols from toxin solutions may be undertaken to test antidotes or vaccines in experimental animals. These are extremely hazardous operations that should only be conducted after extensive validation of equipment and personnel, using non-toxic simulants. Aerosol exposure of animals should be done in a certified Class III BSC or hoodline. While removing exposed animals from the hoodline, and for required animal handling during the first 24 h after exposure, workers should take additional precautions, including wearing protective clothing (e.g., disposable Tyvek suit) and appropriate respiratory protection. To minimize the risk of dry toxin generating a secondary aerosol, areas of animal skin or fur exposed to aerosols should be gently wiped with a damp cloth containing water or buffered cleaning solution before the animals are returned to holding areas.

For high-risk operations involving dry forms of toxins, intentional aerosol formation, or the use of hollow-bore needles in conjunction with amounts of toxin estimated to be lethal for humans, consideration should be given to requiring the presence of at least two knowledgeable individuals at all times in the laboratory.[7]

DECONTAMINATION AND SPILLS

Toxin stability varies considerably outside of physiological conditions depending upon the temperature, pH, ionic strength, availability of co-factors and other characteristics of the surrounding matrix. Literature values for dry heat inactivation of toxins can be misleading due to variations in experimental conditions, matrix composition, and experimental criteria for assessing toxin activity. Moreover, inactivation is not always a linear function of heating time, and some protein toxins possess a capacity to re-fold, and partially reverse inactivation caused by heating. In addition, the conditions for denaturizing toxins in aqueous solutions are not necessarily applicable for inactivating dry, powdered toxin preparations.

General guidelines for laboratory decontamination of selected toxins are summarized in Tables 1 and 2, but inactivation procedures should not be assumed to be 100% effective without validation using specific toxin bioassays. Many toxins are susceptible to inactivation with dilute sodium hydroxide (NaOH) at concentrations of 0.1-0.25N, and/or sodium hypochlorite (NaOCl) bleach solutions at concentrations of 0.1-0.5% (w/v). Use freshly prepared bleach solutions for decontamination; undiluted, commercially available bleach solutions typically contain 3-6% (w/v) NaOCl.

Depending upon the toxin, contaminated materials and toxin waste solutions can be inactivated by incineration or extensive autoclaving, or by soaking in suitable decontamination solutions (See Table 2). All disposable material used for toxin work should be placed in secondary containers, autoclaved and disposed of as toxic waste. Contaminated or potentially contaminated protective clothing and equipment should be decontaminated using suitable chemical methods or autoclaving before removal from the laboratory for disposal, cleaning or repair. If decontamination is impracticable, materials should be disposed of as toxic waste.

In the event of a spill, avoid splashes or generating aerosols during cleanup by covering the spill with paper towels or other disposable, absorbent material. Apply an appropriate decontamination solution to the spill, beginning at the perimeter and working towards the center, and allow sufficient contact time to completely inactivate the toxin (See Table 2).

Decontamination of buildings or offices containing sensitive equipment or documents poses special challenges. Large-scale decontamination is not covered explicitly here, but careful extrapolation from the basic principles may inform more extensive clean-up efforts.

SELECT AGENT TOXINS

Due diligence should be taken in shipment or storage of any amount of toxin. There are specific regulatory requirements for working with toxins designated as a “Select Agent” by the DHHS and/or the USDA. Select Agents require registration with CDC and/or USDA for possession, use, storage and/or transfer. Importation of this agent may require CDC and/or USDA importation permits. Domestic transport of the agent may require a permit from USDA/APHIS/VS. A DoC permit may be required for the export of the agent to another country. See Appendix C for additional information.

TABLE 1 PHYSICAL INACTIVATION OF SELECTED TOXINS

TOXIN	STEAM AUTOCLAVE	DRY HEAT (10 MIN)	FREEZE-THAW	GAMMA IRRADIATION
Botulinum neurotoxin	Yes[a]	> 100ºC[b]	No[c]	Incomplete[d]
Staphylococcal Enterotoxin	Yes[e]	> 100ºC; refolds[f]	No[g]	Incomplete[h]
Ricin	Yes[i]	> 100ºC[i]	No[j]	Incomplete[k]
Microcystin	No[l]	> 260ºC[m]	No[n]	ND
Saxitoxin	No[l]	> 260ºC [m]	No[n]	ND
Palytoxin	No[l]	> 260ºC[m]	No[n]	ND
Tetrodotoxin	Nol	> 260ºC[m]	No[n]	ND
T-2 mycotoxin	No[l]	> 815ºC[m]	No[n]	ND
Brevetoxin (PbTx-2)	No[l]	> 815ºC[m]	No[n]	ND

Table 1 Notes: ND indicates "not determined" from available decontamination literature. [a] Steam autoclaving should be at ≥121°C for 1 h. For volumes larger than 1 liter, especially those containing *Clostridium botulinum* spores, autoclave at ≥121°C for 2 h to ensure that sufficient heat has penetrated to kill all spores.[8,9] [b] Exposure to 100°C for 10 min. inactivates BoNT. Heat denaturation of BoNT as a function of time is biphasic with most of the activity destroyed relatively rapidly, but with some residual toxin (e.g., 1-5%) inactivated much more slowly.[10] [c] Measured using BoNT serotype A at -20°C in food matrices at pH 4.1-6.2 over a period of 180 days.[11] [d] Measured using BoNT serotypes A and B with gamma irradiation from a ^{60}Co source.[12,13] [e] Protracted steam autoclaving, similar to that described for BoNT, followed by incineration is recommended for disposal of SE-contaminated materials. [f] Inactivation may not be complete depending upon the extent of toxin re-folding after denaturation. Biological activity of SE can be retained despite heat and pressure treatment routinely used in canned food product processing.[14] [g] SE toxins are resistant to degradation from freezing, chilling or storage at ambient temperature.[15] Active SEB in the freeze-dried state can be stored for years. [h] References[15,16] [i] Dry heat of ≥100ºC for 60 min in an ashing oven or steam autoclave treatment at >121ºC for 1 h reduced the activity of pure ricin by >99%.[17] Heat inactivation of impure toxin preparations (e.g. crude ricin plant extracts) may vary. Heat-denatured ricin can undergo limited refolding (<1%) to yield active toxin. [j] Ricin holotoxin is not inactivated significantly by freezing, chilling or storage at ambient temperature. In the liquid state with a preservative (sodium azide), ricin can be stored at 4ºC for years with little loss in potency. [k] Irradiation causes a dose-dependent loss of activity for aqueous solutions of ricin, but complete inactivation is difficult to achieve; 75 MRad reduced activity 90%, but complete inactivation was not achieved even at 100 MRad.[18] Gamma irradiation from a laboratory ^{60}Co source can be used to partially inactivate aqueous solutions of ricin, but dried ricin powders are significantly resistant to inactivation by this method. [l] Autoclaving with 17 lb pressure (121-132 ^{o}C) for 30 min failed to inactivate LMW toxins.[17,19] All burnable waste from LMW toxins should be incinerated at temperatures in excess of 815°C (1,500 ^{o}F). [m] Toxin solutions were dried at 150 ^{o}C in a crucible, placed in an ashing oven at various temperatures for either 10 or 30 min, reconstituted and tested for concentration and/or activity; tabulated values are temperatures exceeding those required to achieve 99% toxin inactivation.[17] [n] LMW toxins are generally very resistant to temperature fluctuations and can be stored in the freeze-dried state for years and retain toxicity.

TABLE 2
CHEMICAL INACTIVATION OF SELECTED TOXINS

TOXIN	NAOCL (30 MIN)	NAOH (30 MIN)	NAOCL + NAOH (30 MIN)	OZONE TREATMENT
Botulinum neurotoxin	> 0.1%[a]	>0.25 N	ND	Yes[b]
Staphylococcal enterotoxin	> 0.5%[c]	>0.25 N	ND	ND
Ricin	> 1.0%[d]	ND	>0.1% + 0.25N [e]	ND
Saxitoxin	≥ 0.1%[e]	ND	0.25% + 0.25N [e]	ND
Palytoxin	≥ 0.1%[e]	ND	0.25% + 0.25N [e]	ND
Microcystin	≥ 0.5%[e]	ND	0.25% + 0.25N [e]	ND
Tetrodotoxin	≥ 0.5%[e]	ND	0.25% + 0.25N [e]	ND
T-2 mycotoxin	≥ 2.5%[e,f]	ND	0.25% + 0.25N [e]	ND
Brevetoxin (PbTx-2)	≥ 2.5%[e,f]	ND	0.25% + 0.25N [e]	ND

Table 2 Notes: ND indicates "not determined" from available decontamination literature.[a] Solutions of NaOCl (≥0.1%) or NaOH (>0.25 N) for 30 min inactivate BoNT and are recommended for decontaminating work surfaces and spills of *C. botulinum* or BoNT. Chlorine at a concentration of 0.3-0.5 mg/L as a solution of hypochlorite rapidly inactivates BoNT (serotypes B or E tested) in water.[20] Chlorine dioxide inactivates BoNT, but chloramine is less effective.[21 b] Ozone (>2 mg/L) or powdered activated charcoal treatment also completely inactivate BoNT (serotypes A, B tested) in water under defined condition.[20,22 c] SEB is inactivated with 0.5% hypochlorite for 10-15 mi.[23 d] Ricin is inactivated by a 30 min exposure to concentrations of NaOCl ranging from 0.1-2.5%, or by a mixture of 0.25% NaOCl plus 0.25 N NaOH.[17] In general, solutions of 1.0% NaOCl are effective for decontamination of ricin from laboratory surfaces, equipment, animal cages, or small spills.[e] The minimal effective concentration of NaOCl was dependent on toxin and contact time; all LMW toxins tested were inactivated at least 99% by treatment with 2.5% NaOCl, or with a combination of 0.25% NaOCl and 0.25N NaOH.[17 f] For T-2 mycotoxin and brevetoxin, liquid samples, accidental spills, and nonburnable waste should be soaked in 2.5% NaOCl with 0.25% N NaOH for 4 h. Cages and bedding from animals exposed to T-2 mycotoxin or brevetoxin should be treated with 0.25% NaOCl and 0.025 N NaOH for 4 h. Exposure for 30 min to 1.0% NaOCl is an effective procedure for the laboratory (working solutions, equipment, animal cages, working area and spills) for the inactivation of saxitoxin or tetrodotoxin. Decontamination of equipment and waste contaminated with select brevetoxins has been reviewed.[19]

Alternate methods of chemical decontamination: 1 N sulfuric or hydrochloric acid did not inactivate T-2 mycotoxin and only partially inactivated microcystin-LR, saxitoxin, and brevetoxin (PbTx-2). Tetrodotoxin and palytoxin were inactivated by hydrochloric acid, but only at relatively high molar concentrations. T2 was not inactivated by exposure to 18% formaldehyde plus methanol (16 h), 90% freon-113 + 10% acetic acid, calcium hypochlorite, sodium bisulfate, or mild oxidizing.[17] Hydrogen peroxide was ineffective in inactivating T-2 mycotoxin. This agent did cause some inactivation of saxitoxin and tetrodotoxin, but required a 16 h contact time in the presence of ultraviolet light.

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