

RISKS ASSOCIATED WITH LIQUID NITROGEN CRYOGENIC STORAGE SYSTEMS

Karen B. Byers

Dana Farber Cancer Institute, Boston, Massachusetts

ABSTRACT

Laboratory staff should be aware of the potential for personnel exposure and cross-contamination of improperly stored samples in liquid nitrogen cryogenic storage systems. Published reports indicate that two viruses, Hepatitis B and Vesicular Stomatitis Virus, retain infectivity after suspension in liquid nitrogen. This information confirms the standard biosafety recommendations on the use of personal protective equipment when adding or removing samples from a cryogenic storage system, and on the need to decontaminate equipment when it is being decommissioned or repaired.

Hepatitis B Virus Transmissions

A literature search revealed no laboratory-acquired infections attributed to cryogenic storage systems. However, there is a published report that Hepatitis B Virus (HBV) retained infectivity for over two years when suspended in liquid nitrogen.⁽⁵⁾ In one British hospital, there were six cases of acute HBV infection reported to infection control among multiply transfused patients undergoing cytotoxic treatment. All six patients were seronegative for HBV when treatment was initiated. A study of the transfusion history of the patients revealed no common donor. Other potential common links among the cases were evaluated and eliminated. The times the patients were actually admitted to the hospital did not overlap, (one patient had been treated in a different clinic); and the patients had no personal contact with each other. Even the healthcare workers who cared for the patients were ruled out as a common factor. Only one common link was identified—the harvested bone marrow or peripheral stem cells were stored in the same cryogenic tank.

Cytotoxic treatment involves administration of toxic doses of chemotherapeutic drugs, with the patients' own bone marrow or peripheral-blood stem cells being stored for possible re-infusion. Since a high rate of cell viability on thawing is

critical, the standard for this type of cell storage is a heat-sealed bag of blood cells stored in the liquid phase of cell storage tank.

A newly commissioned tank was used to store the source patient's blood; subsequently, over a two-year period, the bags from the other five patients were added to this tank. It was known that a bag of the source patient's blood had leaked during cryogenic storage. To investigate the potential for HBV transmission, the authors allowed the suspect tank to thaw and analyzed the resulting aqueous liquid. Human DNA sequences and Hepatitis B surface antigen (1 mg/ml) were detected. Polymerase chain reaction studies indicated that the residue from the tank contained Hepatitis B sequences identical to that of the four patients' blood samples available for comparison. (No samples from patient #5 were available for study).

But how was HBV transmitted from the source patient to the other patients? The two remaining bags of the source patient's blood, which appeared to be intact, were removed from liquid nitrogen storage. The bags split open as they thawed; apparently, liquid nitrogen had penetrated the heat seals of the bags. The authors hypothesize that the HBV contaminated the other bags during storage and then was introduced to the patients during re-infusion. Since the type of blood bag used was defective, the ports on the bags could have become contaminated, or the contents of the bag may have come into direct contact with the liquid nitrogen.

This particular type of cryogenic storage bag was recalled by a HAZARD WARNING issued by the UK Medical Services directorate, and this product is no longer used for clinical cryogenic storage. The critical point for biosafety professionals, which has not been previously documented, is that Hepatitis B virus which had leaked directly into liquid nitrogen retained infectivity for a two-year period.

Vesicular Stomatitis Virus Survival

Schering scientists discovered that a glass vial of

Vesicular Stomatitis Virus (VSV) had shattered during liquid nitrogen storage.(3) Since a literature search at that time did not indicate whether the VSV could potentially retain infectivity after suspension in liquid nitrogen, an experiment was conducted. Liquid nitrogen aliquots were aseptically removed from the storage tank and were allowed to evaporate in their container in a biosafety cabinet. Small volumes of saline were used to wash the containers; VSV was detected in the washes. To confirm the finding, VSV was added directly to volumes of liquid nitrogen; there was no loss of infectivity.

Implications for Biosafety Professionals

Review of storage conditions where biohazardous samples are maintained in liquid nitrogen is essential to a biosafety audit. More research is required to define the classes of viruses and/or other microorganisms that retain infectivity when inadvertently suspended in liquid nitrogen. The data available on two viruses may help to educate staff on the need for careful use of liquid nitrogen cryogenic storage systems. Proper personal protective equipment when adding or removing samples from such systems is required.(1) An understanding of the limitations of the type of cryovial used minimizes the potential for contamination of the liquid nitrogen.

An excellent resource on the mechanics of liquid nitrogen cryogenic storage is the Cryopreservation Manual distributed by NALGE Nunc International Corp.(4) Despite such publications, researchers often fail to follow manufacturer recommendations. The most common container for cryogenic storage is the plastic cryovial. These are supplied in packages marked "FOR VAPOR STORAGE ONLY", however, this warning is often ignored. Using the vial in the liquid phase of the tank increases the risk of exploding vials and cross-contamination of samples. Rather than paraphrase information related to the safety considerations associated with cryogenic storage, they are reproduced from the NALGENE® Cryopreservation Manual below.(4)

"Safety precautions must be observed throughout the preservation and maintenance process. All work with hazardous cultures should be performed under proper

containment, and U.S. Public Health Service Biosafety guidelines should be adhered to at all times.(6)

Animal cells may contain adventitious viral agents that require special handling, and all animal cells that have not been thoroughly characterized should be handled at Biosafety Level II. At this level, laboratory staff must have training in handling pathogenic agents and work under the direction of a competent scientist. Access to the laboratory must be limited and biological safety cabinets must be used for large-volume work or when aerosols are generated.(6)

Low-temperature storage of cells presents unique hazards that necessitate safety precautions. Cryogenic temperatures can result in exposure of personnel to extremely cold conditions, and precautions must be taken to protect personnel during operations in liquid nitrogen freezers. Insulated gloves and long-sleeved laboratory coats or other garb protect the skin from exposure. It is extremely important to wear a full-face shield and neck shield when working in the liquid portion of a liquid nitrogen freezer. As noted above, improperly sealed glass ampoules may explode when retrieved from liquid nitrogen. To minimize the risk of potential explosions, place the ampoule or vial in the vapor phase for 24 hours. A face shield that provides neck protection should be mandatory when retrieving vials from liquid nitrogen. The use of NUNC™ CryoFlex™ is also strongly recommended. **See warning**

WARNING:

Do not use vials for storage in the liquid phase of liquid nitrogen unless correctly sealed in NUNC™ CryoFlex™ Tubing (Cat. No. 343958). Improper use may cause entrapment of liquefied nitrogen inside the vial and lead to pressure build-up, resulting in possible explosion or biohazard release. Use appropriate safety procedures as outlined in this manual when handling and disposing of vials.

Special precautions must always be taken when working with hazardous biological materials at liquid nitrogen temperatures.

Always thaw and open vials containing hazardous material inside a biological safety cabinet. Be prepared for exploding and leaking ampoules/vials. Broken ampoules in a liquid nitrogen freezer are a potential source of contamination (3), and contaminants may survive, despite the extremely cold temperatures. When a liquid nitrogen freezer becomes contaminated, the entire unit should be decontaminated after warming to room temperature. When closing down a liquid nitrogen freezer that is not obviously contaminated, remove all material to be retained, warm the unit to room temperature and disinfect it prior to further handling.”(4)*

Limitations of Liquid Nitrogen Storage Systems

The temperature at which frozen materials are stored affects cell viability. The lower the temperature, the longer the cells can be recovered. Ultimate stability of mammalian cells cannot be assured unless the cells are maintained below -130° Centigrade (C). Liquid nitrogen units that provide all-vapor storage are ideal, but the working temperature at the top of the unit must be maintained below -130° C. This requires careful adjustment of the liquid level. The *Cryopreservation Manual* states that this can be achieved in most working units, “however, the design of some models means the amount of liquid nitrogen necessary to attain the proper working temperature will reduce the amount of usable storage space.”(4) Some researchers may feel that raising the level of liquid nitrogen will improve viability because the cells will be kept at a lower temperature; it is important to explain that storing vials designed for vapor storage in the liquid phase may contaminate the liquid nitrogen and cross-contaminate stored samples.

One manufacturer provides a plastic sleeve for cryovials stored on a wand; encased in this sleeve; the cryovials may then be submerged in the liquid phase. For cryovials stored in boxes, some researchers leave the bottom spaces of their storage rack empty. This method is satisfactory only as long the level of liquid nitrogen does not exceed the level at which samples are stored. Two problems with this method are that: 1) adequate control of the liquid level may be difficult in manual-fill systems and 2) maintaining this system requires the

cooperation of the users. A better option for biohazardous sample storage is an automated-fill liquid nitrogen system with a platform between the liquid and vapor phase. The vapor platform is offered by manufacturers and relatively inexpensive. Shorter racks are purchased to sit on top of the vapor platform. After setting the liquid nitrogen level to just below the platform, the system controls can be locked to prevent tampering. This measure will provide the maximum achievable control of a liquid nitrogen storage system and, given the potential for viral contamination, may be the best solution for biohazardous sample storage.

The standard operating procedure (SOP) for a cryogenic storage unit should include information on the decontamination procedures to be followed before service or decommissioning. The SOP should include informing the Laboratory Director if problems occur such as overfilling of the tank beyond the required level or dropping of vials into the liquid phase of the cell storage system.

Mechanical Freezer Systems

New developments in ultralow freezer technology may allow mechanical freezers to replace liquid nitrogen for cryogenic storage in research and clinical applications.(2) The new compressor technology provides air-phase storage at -140 and -150° C without the need for liquid nitrogen. According to the manufacturer, mechanical freezer systems will actually be less expensive than liquid nitrogen cell storage systems when one factors in costs associated with the rental and delivery of liquid nitrogen tanks.(2) This is an exciting new development. However, for most research programs, it will be years before replacement is considered. Until new mechanical freezer technology replaces liquid nitrogen storage systems, biosafety professionals must continue to evaluate standard operating procedures for the use of the numerous liquid nitrogen storage tanks in laboratories. This article was submitted to raise awareness of the biohazard potential and usage limitations of cryogenic storage systems.

*NOTE: Information reprinted with permission from Nalgene NUNC. Use of product names does not imply endorsement by the *Journal of the American Biological Safety Association*, American Biological Safety Association, or the author.

ACKNOWLEDGEMENT

Lynn Harding provided invaluable assistance with the manuscript.

REFERENCES

Hunt, D. "Human Immunodeficiency virus type 1 and Other Blood-Borne Pathogens," p. 51, in Fleming, D.O., J.H. Richardson, J. Tulis, D. Vesley, eds. Laboratory Safety, Second Edition. Principles and Practices. 1988. ASM Press, Washington, DC.

Lehman, R.D. 1996. "A Mechanically Refrigerated Cryogenic Freezer for Air-phase Storage of Biologicals at -150° Centigrade without Liquid Nitrogen." American Laboratory, 25-30.

Schafer, T.W., J. Everett, G. Silver, P.E. Came. 1976. "Biohazard Virus-Contaminated Liquid Nitrogen." Science 191:24-26.

Simione, F. 1998. American Type Culture Collection in cooperation with NALGE Nunc International Corp. CRYOPRESERVATION MANUAL.

Tedder, R.S., M.A. Zuckerman, A.H. Goldstone, A.E. Hawkins, A. Fielding, E.M. Briggs, D. Irwin, S. Blair, A.M. Gorman, K.G. Patterson, D.C. Linch, J. Hepstonstall, N.S. Brink. 1995. "Hepatitis B Transmission from Contaminated Cryopreservation Tank." Lancet 346:137-139.

U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, and National Institutes of Health, Bethesda, Maryland. 1988. Biosafety in Microbiological and Biomedical Laboratories.

IMPORTANT..... IMPORTANT..... IMPORTANT..... IMPORTANT

A change of address notice should be sent at least six weeks in advance to the ABSA National Office to ensure that all mailings, including the journal and newsletter, will reach you. ABSA is not responsible for misrouted mail as a result of insufficient notification of an address change. Undelivered copies resulting from an insufficient address change notification will not be replaced, but single issues may be purchased at the single issue price.

CHANGE OF ADDRESS FORM

Name _____

Old Address _____

Old Phone Number _____

NEW ADDRESS _____

CITY _____ STATE _____ ZIP +4 _____

NEW PHONE NUMBER _____

E-MAIL ADDRESS _____

Effective Date _____