From Bench to Cageside: Risk Assessment for Rodent Pathogen Contamination of Cells and Biologics

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Abstract

Many newly developed animal models involve the transfer of cells, serum, or other tissue-derived products into live rodents. These biologics can serve as repositories for adventitious rodent pathogens that, when used in animal studies, can alter research outcomes and result in endemic outbreaks. This review includes a description of some of the biologics that have inadvertently introduced infectious agents into in vivo studies and/or resulted in endemic outbreaks. I also discuss the points of potential exposure of specific biologics to adventitious rodent pathogens as well as the importance of acquiring a complete developmental and testing history of each biologic introduced into a barrier facility. There are descriptions of specific cases of mycoplasma and lactate dehydrogenase–elevating virus (LDHV), two of the most common organisms that contaminate cells and cell byproducts. The information in this article should help investigators and animal resource program personnel to perform an appropriate risk assessment of biologics before their use in in vivo studies that involve rodents.

Introduction

Numerous animal models are produced by the transfer of exogenous cells or cell/tissue-derived products (biologics) into rodents, practices that increase the risk of introducing contaminants into the barrier. To reduce these risks, most institutions implement strict policies that require testing of biologics for the presence of adventitious infectious agents before their use within the barrier. These tests are typically available through commercial services that use polymerase chain reaction (PCR) assays to detect the presence of nucleic acid sequences of viruses or mycoplasma (Bauer et al. 2004). Unnecessary testing of samples can waste time and resources, but lack of adherence to strict barrier practices (including the prudent testing of biologics) can leave institutions vulnerable to costly expenditures for remediation of contamination. Thus it is important for investigators and those that manage laboratory animal resources to understand and assess the risks associated with the use of cell lines and biologics.

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The potential of infectious contaminants to confound in vivo studies drives the need for barrier practices and risk assessment. The effects of numerous rodent pathogens on biologic systems and on research are described in other articles in this issue (Besselsen et al. 2008; Mahabir et al. 2008; Watson 2008) and elsewhere (Lindsey et al. 1991). Institutions go to great lengths to prevent rodents that may be infected with rodent pathogens from entering their animal facilities, so it is certainly appropriate to accord equal diligence to items that enter the barrier in vials and test tubes. Similar to the importation of live rodents, cell lines and biologics derived from colonies that are stringently monitored for adventitious infectious agents are at lower risk of contamination than those from colonies of unknown status.

Cell Lines

The "compatibility" of an infectious agent with the cell line or biologic also influences possible outcomes of contamination. Viruses that can infect and replicate in the cultured cell line will be able to maintain a higher titer through several passages (generations of cell harvests and seeding) and this in turn will affect the concentration of the viral contaminant when the cells are used in vivo. Agents that have limited ability to replicate in their cohabiting cell line pose less of a threat, because the concentration of the contaminating agent remaining in the culture is dependent on the number of passages since contamination and the agents' ability to survive in the culture media. The in vitro (and in vivo) characteristics of any viral infection are likely to be highly strain dependent. Last, the response of the recipient may also influence the ability of the infectious agent to cause disease and spread. More often than not, cell lines are introduced into immunocompromised mice, providing greater opportunities for any contaminating infectious agent to incubate and multiply in the absence of adversarial host response.

Tumor cell lines are the most common biologic brought into the animal barrier facility. There are numerous reports of contamination of tumor cell lines with lymphocytic choriomeningitis virus (LCMV; Bhatt et al. 1986; Dykewicz et al. 1992), mouse hepatitis virus (MHV[1]; Takakura et al.

[1]Abbreviations used in this article: BMM, basement membrane matrix; LDHV, lactate dehydrogenase–elevating virus; MHV, mouse hepatitis virus; MPV, mouse parvovirus

2000), lactate dehydrogenase–elevating virus (LDHV[1]; Chen and Plagemann 1997), mouse parvovirus (MPV[1]; Garnick 1996), and retroviruses (Suzuki et al. 1977). Both the zoonotic potential (Bowen et al. 1975) and a recently reported endemic infection (Ike et al. 2007) of LCMV underscore the importance of continued surveillance for this organism. MHV and MPV outbreaks remain the most common causes of endemic infections in laboratory colonies (Jacoby and Lindsey 1998; Schoondermark-van de Ven et al. 2006), but the percentage of outbreaks attributable to MHV and MPV contamination of transplanted cells is unknown. Endogenous retroviruses are present in all murine cell lines, and their activation may affect some cancer and/or virus studies (Aoki et al. 1977; Suzuki et al. 1977). Because of their ubiquitous nature, cell lines are rarely monitored for the presence of retroviruses, except for use in commercial production of biologics (see Shepherd et al. 2003 for further information).

Cell Lines at Higher Risk of Exposure

It is not necessary to test a cell line that has never been exposed to rodent tissues or rodent-derived substrates for rodent pathogens. However, unless a detailed and complete history of the cell line is available from the progenitor and subsequent handlers, there may have been opportunities for exposure that would put the cell line at risk for contamination. Potential points for rodent pathogen exposure can come early in a cell line's development. For example, primary mouse fibroblast cells and some specifically selected cell lines produce undefined factors that are conducive to cell growth and are often necessary to maintain the viability of primary cultures (Lazaro et al. 1998; Orlik and Altaner 1988). Once the primary cells become established, these "feeder cells" are no longer needed and their previous presence in the culture can be easily forgotten. In some cases conditioned media, in which the feeder cells have been cultured, can substitute for the cells themselves and this substrate may also carry contaminants (Bazin and Lemieux 1989).

Cell lines that present increased risks for potential contamination with rodent pathogens include stem cells, hybridomas, and other cancer cell lines. Stem cells are being increasingly studied for potential application as therapeutics in almost every field of biomedical research (Taupin 2007). Risks may increase with stem cells that are derived from rodents and/or that require the presence of rodent-derived feeder cells (stromal cells/fibroblasts) for culture maintenance (Hirashima et al. 1999). Investigations are also under way to define the growth factors and cytokines that control stem cell growth and differentiation in vitro (Kouro et al. 2007). The rederivation of these rodent-derived reagents offers an additional avenue for the introduction of infectious contaminants. As Nicklas and Weiss report (2000), embryonic stem cells from institutes with high health monitoring standards have a lower risk of contamination; the authors were unable to detect viral contaminants in the 46 stem cell lines they analyzed. However, it is important to remain vigilant, as Kyuwa (1997) and Okumura and colleagues (1996) demonstrated that mouse hepatitis virus replication can persist in embryonic stem cells.

Hybridoma cell lines are susceptible to contamination from four sources. The cell lines are the product of the fusion of splenocytes, typically from antigen-stimulated rodents, and myeloma cell lines, also typically derived from mice; infection in either of these cell lines is likely to cause contamination of the resulting hybridoma cell line. The third source of contamination of hybridomas is feeder cells (mouse fibroblasts) or conditioned media added to postfusion cells to increase survival during clonal expansion (Yokoyama et al. 2006). Last, some researchers believe that it is possible to restore high antibody production from failing hybridoma cells by growing them in the peritoneal cavity of mice and returning the cells to culture. This procedure exposes the cells to yet another mouse colony that could be infected and can therefore result in contamination. In addition, MHV and MPV acquired in the mouse can continue to infect and replicate in cells of lymphoid lineage in vitro, making hybridomas more vulnerable to persistent contamination with these organisms (Lamontagne et al. 1989; Previsani et al. 1997).

Most cancer cell lines are taken from their tissue of origin and dispersed in enriched media to establish a cell line. If these cell lines are derived from nonrodent sources and maintained in standard culture media there is minimal risk of rodent viral contamination. In some cases, cells from a tumor may not adapt well to the in vitro environment and so are implanted from the human (or other species) directly into immunocompromised mice (Morton and Houghton 2007). Cells that are supported by the mouse's system will expand and provide a source for repeated trials to optimize conditions for in vitro growth. Cell lines that do not adapt to in vitro conditions may need to be maintained by repeated passage in the mouse (Sobel and Sadar 2005). This repeated and often prolonged exposure to different mice increases the risk of contamination for these cells. Established tumor cell lines may also be taken out of culture and injected in rodents in order to develop metastatic variants; the cells are injected intravenously and reisolated from target organs of interest (lung, brain, liver, bone marrow) (Kitamura et al. 2000; Victor et al. 1999). Multiple cycles of reisolation/injection may be necessary to develop the desired characteristics. The cells are then typically returned to culture and maintained in vitro until they are needed for an in vivo experiment.

Incidence of Contaminants in Cell Lines

The most recent report of a comprehensive survey of infectious rodent agents in cell lines was by researchers at a Japanese institute in 2000 (Nakai et al. 2000). The only viral contaminant they found was lactate dehydrogenase–elevating virus (LDHV), and it was present only in cell lines

that had been propagated in mice (9 of 14); none of the other 82 cell lines maintained in vitro had evidence of viral contamination. In a similar, earlier survey at a German institute, Nicklas and colleagues (1993) reported contamination of 25% of cell lines tested, with LDHV present in 17.8% of the lines tested. As suggested by these studies and a review by Blank and colleagues (2004), the incidence of infectious rodent agents in cell lines has markedly decreased over the last 35 years. Records from our institution covering the last 3 years support this assumption, as none of the 153 cell lines that we have imported from vendors and other institutions have tested positive by polymerase chain reaction (PCR) diagnostics for any of the rodent viruses on the RADIL-IMPACT II Profile (RADIL; Columbia, MO) (personal communication with Marie-Louise Miller, MedImmune, Inc., Gaithersburg, MD, August 2007).

The decreased incidence of contaminants in cell lines is likely due to three factors: aggressive health monitoring programs of rodent facilities resulting in decreased opportunities for contamination of newly developed cell lines; increased awareness, testing, and culling of cell lines; and, during the late 1990s, the replacement of the cumbersome MAP assay with the quicker PCR-based diagnostic assays for cell line testing. The two studies by Nakai and colleagues and Nicklas and colleagues (discussed above) used the mouse antibody protection (MAP) assay.

Criteria for importing a cell line into an animal barrier facility should be somewhat similar to those for importing rodents: strict barrier controls do not allow the admission of mice with an unknown history or health monitoring record into the general colony population, and the same should hold true for cell lines. Because cell lines can be cultured for many years in a laboratory, frozen for decades in liquid nitrogen, and exchanged several times, their testing records and exposure history are likely to be incomplete. Unlike typical rodent barrier facilities, it is unnecessary and/or impractical to periodically test cell lines that have been exclusively maintained in culture for rodent viruses. As stated earlier, not all cell lines need to be tested; exceptions should be made only if a complete history of testing is available or there is a high level of confidence that exposure to rodents or rodent-derived reagents has not occurred.

Mycoplasma

Mycoplasmas, which can easily enter culture flasks, are the nemesis of every technician that maintains cell cultures. These organisms are ubiquitous and can be carried on the skin or in the upper respiratory tract of humans. Because *Mycoplasma* contamination of cell lines can wreak havoc on cellular studies, high-quality laboratories periodically test cultures for this pathogen. Identification of *Mycoplasma* in cell lines is also of concern to those who perform in vivo studies because of the well-documented effects of contamination on cell growth and the immune system (Uno et al. 1990; Ushio et al. 1995). Caution is further warranted as the administration of *Mycoplasma*-contaminated cells to immunocompromised mice (and other strains) can result in sepsis and other clinical illnesses (Dodds et al. 2003).

Although cell lines are highly unlikely to be contaminated with rodent mycoplasmas (*M. pulmonis*, *M. rodenticum*) while being cultured in the laboratory, most tests do not differentiate/speciate rodent pathogens from those commonly present in the laboratory environment. Nakai and colleagues (2000) found that 29 of 73 cell lines at their institution were contaminated with *Mycoplasma* sp., although *M. pulmonis* was not found in any of the samples. Endemic infections with *M. pulmonis* have been relatively rare in the 21st century, and thus the risks associated with contamination of cell cultures derived from, or exposed to, barrier-maintained rodents in the last decade are much lower than before.

Although most researchers are aware of the confounding effects, there may be instances in which in vivo application of *Mycoplasma*-contaminated cells is necessary. One such situation occurred at our institution when investigators were unable to reproduce a collaborator's in vivo study results and the only difference between laboratories was that they were using *Mycoplasma*-contaminated cell stocks. In order to determine if this was a variable within a strict timeline, use of these cell lines in our barrier facility was permitted on the condition that they be demonstrably free of *M. pulmonis* or *M. rodenticum*. Although there are no reports of rodent-to-rodent transmission of the common laboratory *Mycoplasma* contaminants, because immunocompromised mice were used, we placed them in a quarantine room and implemented special husbandry and technical procedures to reduce the risk of transmission.

In vitro approaches for decontaminating cell cultures with antibiotics are available, but their ability to rid cultures of *Mycoplasma* contamination is questionable. *Mycoplasma* is resilient, and periodic post-treatment testing is necessary to differentiate reduction (beyond detection) in viral load from cure. It is also advisable to consider the possibility of phenotypic alterations of the cell line after exposure to antibiotics. Alternatively, an old established method for curing a cell line of *Mycoplasma* contamination is to introduce the cells into a rodent, allow its immune system to clear the organism, and reharvest the cells for continued culture (Roseto et al. 1984). This procedure is another example of how cell lines can be exposed to rodent viruses as described in the preceding section.

Biologics

Although cell lines are the most common biologics used in animal research, other animal products merit careful attention. Numerous types of biologic agents are used in live rodent studies: bacteria, viruses, parasites, serum and serum-derived products, cytokines/hormones/growth factors, antibodies/antibody fragments, DNA/RNA, basement membrane matrices (Matrigel®), and mouse gametes and em-

bryos. The risk of contamination in these agents is largely dependent on the material's exposure to rodents that are either infected or of unknown status.

Institutions vary in the types of biologics that are screened before in vivo use. A quick review of websites (searching on "IACUC, cells, policy, rodent, viruses") revealed that of twenty institutions that posted documents or policies restricting the use of cell lines in animals, only seven included requirements for testing of other cell-associated or biological material. Although there are many shortcomings to interpreting data gathered this way, the exercise suggests that a significant number of institutions may not monitor for these potential sources of rodent virus contamination. Additionally, some biologics entering an animal barrier facility may not be reviewed or deemed a threat to barrier integrity because the possibility of their having rodent-associated origins was never considered.

An example of an inconspicuous biologic is basement membrane matrix (BMM[1]) protein extracts, commonly used in tumor growth studies and available from vendors that distribute a variety of laboratory reagents. Early in 2007, our institution received reports from a contract laboratory that some of our tumor studies would be delayed because lactate dehydrogenase–elevating virus (LDHV) contamination was discovered in three lots of BMM. Surveys of other institutions revealed that five lots of BMM showed evidence of LDHV contamination. Although the supplier reported using a contract laboratory that implemented a sentinel health monitoring program, LDHV remained undetected in the colony that housed the mice from which the BMM-producing cells were harvested. The fact that LDHV does not readily spread to sentinels (Lindsey et al. 1991) likely explains, in part, why this organism escaped detection and why it is therefore commonly found in cells and biologics. This incident clearly revealed that although the use of sentinels in health monitoring programs may reduce the risks of viral contamination to animals and biologics, agents such as LDHV require monitoring directly in the sample (mouse or biologic) itself. This organism may adversely affect research involving infected mice; fortunately, because its transmission generally requires direct contact, LDHV contamination in a colony does not require extensive decontamination measures as compared to contamination with mouse hepatitis virus or mouse parvovirus.

Animal model studies frequently call for the use of infectious agents, which are another potential source of rodent virus contamination that may be easily overlooked. For example, one mouse study used toxoplasma that turned out to be contaminated with lymphocytic choriomeningitis virus, bringing into question several previous studies performed with this organism (Grimwood 1985). Bacteria and viruses may be passed through rodents in order to increase their virulence and then returned to culture for maintenance (Al-Hello et al. 2005; Mase et al. 2006; Peters and Paterson 2003). Again, the possibility of these stocks becoming contaminated depends on the infectious status of the surrogate rodents. Similar to cell lines, knowledge of where the agents have been is crucial in assessing the risks imposed.

Contaminated mouse serum can also be the source of endemic infections, as demonstrated by a research colony outbreak of ectromelia that was reported to result from injection of mice with pooled, imported mouse sera (Lipman et al. 2000). Pooling of serum from mice is a typical means of obtaining the quantities and uniformity needed for experimental purposes. Because this practice potentially widens the distribution of infectious agents, there are increased risks associated with pooled serum samples. Most serum transfer protocols are likely to involve mice in the same facility, and in these instances the risk of introducing new contaminants is negligible. However, there are some current models, such as the K/BxN serum transfer model of rheumatoid arthritis, in which serum production can be contracted off-site, stored, and imported (Kouskoff et al. 1996). In this situation, and others that involve off-site production of biologics for in vivo use, the health status of rodents at the sponsor site may be at risk of exposure to infectious agents from the contracted site. Careful review of the procedures and health surveillance of the contracted institution can help to reduce these risks.

As discussed above, just as hybridoma cell lines are at risk of contamination, so are the antibodies they produce. Antibodies are the most common rodent-derived protein used in biomedical research. When bound to their targets, many of them disrupt specific protein interactions and cellular functions involved in infection, inflammation, or cancer cell growth. Antibodies that show potential as research tools and/or therapeutics are often tested further in mouse models (Harris and Adair 1997). A survey by Nicklas and colleagues (1993) reported LDHV contamination in two monoclonal antibodies (crude ascites), one of which unexpectedly caused an increase in serum interferon levels during an experimental study (Nicklas et al. 1988). Purification procedures are likely to reduce viral load in monoclonal antibody preparations, although studies supporting this hypothesis are lacking. Monoclonal antibodies that are (1) produced strictly by in vitro methods from a hybridoma cell line proven to be negative for rodent viruses and (2) purified over a dedicated column should not require further testing for these agents (although periodic mycoplasma testing of the culture is advisable).

As more institutions acquire the technologies to ship, receive, and manipulate mouse gametes and embryos, the likelihood of these resources transmitting infectious agents between institutions will doubtless increase without close monitoring. Agca and colleagues (2007) demonstrated that mouse parvovirus can be carried in mouse gametes, embryos, and ovarian tissues that originate in naturally infected colonies. The results of their study emphasize that caution is necessary with imported germplasm for use in such applications as embryo transfer, establishment of embryonic stem cell lines, in vitro fertilization, ovary transplantation, and intracytoplasmic sperm injection. In this issue Mahabir

and colleagues (2008) also discuss the contamination risks of germplasm trafficking.

Summary

Contaminants may inadvertently enter an animal facility not only in imported mice infected with rodent viruses but also in vials and test tubes. In order to effectively maintain barrier function, investigators need to be meticulous and forthright about the materials used in their animal studies. Investigators, facility managers, veterinarians, and the institutional animal care and use committee (IACUC) members also need to be familiar with how these biologics (and their components) are derived in order to effectively evaluate the risks of rodent virus contamination. Repeated testing of a cell line or biologic (lot) for infectious rodent agents may not be necessary if accurate and complete records show that there was no exposure to potential contaminating tissues or their derivatives from the time of development or since the date of the last test. But if records are incomplete and/or the history of the material is not completely known, then risk mitigation requires the testing of samples before their use in vivo.

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