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Article *in* Cancer Gene Therapy · January 2003

DOI: 10.1038/sj.cgt.7700536 · Source: PubMed

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Review

Biosafety and product release testing issues relevant to replication-competent oncolytic viruses

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Replication competent oncolytic viruses, like other biological products, are at risk from contamination by bacteria, fungi, mycoplasma and viruses that must be eliminated from the final product. This article reviews the regulatory guidance for the manufacture and testing for oncolytic virus products. A testing strategy covering the testing of cell lines, virus banks, virus harvests and purified product is described.
Cancer Gene Therapy (2002) **9,** 1056–1061 doi:10.1038/sj.cgt.7700536

Keywords: oncolytic; virus; vector; contaminants; testing; safety

Relevant issues

Biological products such as oncolytic viruses, vaccines, gene therapy vectors, and recombinant proteins are at risk from the possibility of contamination by bacteria, fungi, mycoplasma, and viruses that must be eliminated from the final product. For these products, this contamination risk can be minimized by the screening of raw materials used in production, control of the production process, and incorporation of specific contaminant clearing processes wherever possible.

Cell lines used to produce the oncolytic viruses can potentially be contaminated with adventitious viral and mycoplasma contaminants. Prior to the institution of regulatory requirements in 1962, millions of people worldwide were inoculated with poliovirus and adenovirus vaccines, manufactured in rhesus monkey kidney cells, that were contaminated with SV40.[1] Mycoplasma contamination has been detected in 15–35% of cell lines deposited in some cell culture collections.[2] Although the manufacture of recombinant proteins and vaccines from cell lines has a good safety record, there have been reports of the contamination of production processes and viral vaccines by viruses most probably derived from contaminated media components and fetal calf sera.[3–7] Porcine parvovirus has been isolated from commercial trypsin used in passaging of adherent cell lines.[8] In addition to adventitious viral contamination, many cell lines express endogenous retrovirus-like particles[9,10] and batches of human vaccines produced using avian cells have been contaminated with retrovirus-like particles.[11,12] The extent of adventitious and endogenous contamination of cell lines tested in support of clinical studies has been documented.[13]

Received September 12, 2002.

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The integrity of any production system for oncolytic viruses also requires the implementation of identity tests for both the virus and cell stocks to be used in production. Cross-contamination of cell lines has been reported at significant rates.[14]

Regulatory guidance

There is, at present, no specific regulatory guidance relating to the production and testing of replication-competent oncolytic viruses. Vectors engineered to express new genes are covered by the guidance given for gene therapy vectors[15,16] and the principles of testing for adventitious contaminants and cell line identity are covered in earlier documents.[17–19]

The principles to be addressed in the manufacture of sterile products including viral vectors are contained in the European Community Guide to Good Manufacturing Practice[20] and the European Commission has recently revised Annex 13 of this guide covering manufacture of investigational medicinal products produced for clinical studies.[21] This year, the European Commission published a directive relating to Good Clinical Practices that will require products for all clinical trials in the EU to be produced in premises licensed for good manufacturing practice (GMP).[22] Following the bovine spongiform encephalopathy (BSE) epidemic in the UK and the link to variant Creutzfeldt-Jakob Disease (vCJD) in humans, manufacturers using bovine-derived products in their process must also follow the guidance relating to minimizing the risk of transmissible spongiform encephalopathies.[23]

Probably the most relevant set of regulatory documents relating to the testing of replication-competent oncolytic viruses can be found in the sections of the European Pharmacopoeia (EP) dealing with the testing of live human vaccines. The EP has specific sections dealing with the testing of cell substrates used for the production of human vaccines,[24] virus seed lots, virus harvests, and production

control cells.[25] These documents, when interpreted in relation to the other documents mentioned above, can be used as the basis of testing strategy for oncolytic viruses described below.

Manufacturing quality for oncolytic viruses

Testing of a final product cannot, of itself, ensure quality and safety. To achieve the quality objective reliably, there must be a correctly implemented system of quality assurance (QA) that incorporates GMP and quality control (QC). GMP is that part of QA that ensures that the product is consistently produced and controlled to the quality standards appropriate to the intended use of the product. GMP is concerned both with production and QC. The basic requirements of GMP are that:

(1) Manufacturing processes are clearly and systematically defined and reviewed and shown to be capable of consistently manufacturing products of the required quality.
(2) Critical steps of the manufacturing process are validated. For early phase clinical manufacture, this may be restricted to validation of the filling process.
(3) The following facilities are provided:
  (a) appropriately qualified and trained personnel;
  (b) adequate premises and space. For sterile manufacture, this would include appropriately HEPA filtered clean air supply;
  (c) suitable equipment and services;
  (d) correct materials, containers, and labels;
  (e) suitable storage and transport;
  (f) approved procedures and instructions.
(4) Procedures are written in clear, unambiguous language specifically applicable to the facilities provided.
(5) Records are made, which demonstrate that all steps required by the procedures were in fact taken and enable a complete history of a batch to be traced. Records must be retained in a comprehensible and accessible form.
(6) The distribution of the product minimizes any risk to its quality and that a system is available to recall any batch of product.
(7) A QC system is set up, with similar standards relating to personnel, facilities, procedures, and records that deal with the sampling, inspecting, and testing of starting materials; packaging of materials and intermediate, bulk, and finished products; and the appropriate environmental monitoring of the facilities.

Strategy for the testing of oncolytic viruses

For oncolytic virus products, the QC testing of raw materials includes the testing of production cell lines, virus banks, lots of media, sera, and other reagents such as trypsin. The testing of process intermediates such as bulk harvests and bulk-purified products compliments a well-designed and consistent manufacturing process.

Ideally, a manufacturing process for a sterile biological product will incorporate methods for the removal and/or inactivation of contaminants. In contrast to the manufacture of recombinant proteins, when the oncolytic product is a live virus, it is not easy to incorporate processes for the clearing of microbial contaminants that do not inactivate or remove the product as well. For unenveloped viral vectors such as adenovirus, manufacturers have used organic solvent treatment to purify the unenveloped virus and to inactivate enveloped viruses.[26] Without a robust method for inactivating adventitious microbial contaminants, more emphasis should be put on the testing of raw materials and process intermediates and on the incorporation of more exacting standards of GMP.

Any testing strategy will also be influenced by the phase of clinical development, the patient population, and clinical indication being treated. Most regulatory guidance documents concentrate on the issues that should be addressed prior to product licensing. The expectations of regulatory authorities in regard to process control and validation, QC testing, and validation and incorporation of appropriate standards for production will increase throughout the clinical development phase. Developers of oncolytic viral vectors should draw up a development strategy and discuss this with their local regulatory agency prior to applying for clinical trial approval. In *Testing of Cells Used in Production*, the different assays that should be included in a testing strategy will be discussed. Any selected package of tests should be designed with relevance to the stage of clinical development of the product.

Testing of cells used in production

The quality and safety of the cells used in the production of viral vectors are assured by preparing and testing cell banks. Sufficient vials of a Master Cell Bank (MCB; see Ref. [19] for definition) should be prepared to last the potential lifetime of the product. These cells should be tested for identity and the absence of adventitious contaminants. To ensure the absence of low-level contaminants, cells should also be tested after expansion under pilot or production conditions to the limit of *in vitro* cell age used for production.[19] The testing of any Working Cell Bank (WCB) produced from the MCB can be minimal because this bank will be tested after expansion to the limit used for production (Table 1).

As full a history as possible should be generated for the cells that will be used to prepare the MCB. The growth characteristics of the cells and their morphological properties should be examined by light and electron microscopy. DNA fingerprint analysis[27,28] or an alternative method such as isoenzyme analysis[29] should confirm the identity of the cell line. Most regulatory documents related to the production of recombinant proteins or gene therapy vector production require the use of only one identity test; however, the EP requires two identity tests to be applied to cells used for the production of human vaccines.

Human diploid cells such as MRC-5 and WI-8 cells are recognized as being diploid and well characterized. If they

Table 1 Testing of oncolytic viruses cells used in production

Assay	MCB*	WCB†	Cells at the limit of *in vitro* cell age used for production‡	Production control cells§
Morphology	+	+	+	+¶
Identity — isoenzymes	+	+	+	–
DNA fingerprinting	+	+	+	–
Karyology (diploid cell lines)	+	–	+	–
Tumorigenicity	–	–	+	–
Bacterial and fungal contamination	+	+	+	–
Mycoplasma	+	+	+	+
In vitro virus assay (cpe, HA‖)	+	–	+	+
In vivo virus assay#	+	–	+	–
Electron microscopy	+	–	+	–
Specific viruses**	+	–	+	–
Bovine viruses††	+	–	+	+‡‡
Porcine viruses§§	+	–	+	+¶¶
Retroviruses (PCR-based RTase‖‖‖)	+	–	+	–

*MCB: Master Cell Bank.

†WCB: Working Cell Bank.

‡CAL: cells at the limit of *in vitro* cell age used for production.

§Production control cells: cells from the same batch of cells used for production, mock-inoculated, and handled at same time and in same way as production cells.

¶Examine production control cells for freedom from viral cytopathic effects.

‖Cpe: viral cytopathic effects; HA: hemadsorption.

#*In vivo* virus assay: inoculate cell lysate into suckling mice, adult mice, and embryonated eggs (allantoic cavity and yolk sac).

**Specific viruses: if human cells are used for production, they should be tested for specific human viruses by nucleic acid amplification methods. The specific viruses should be a choice of the following relevant viruses: HIV 1 and 2, HTLV I and II, hepatitis B, hepatitis C, cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpes viruses HHV 6, 7, and 8, human polyoma viruses, simian virus 40 (SV40), human parvovirus B19. If rodent cells are used, then these should be screened for rodent viruses in mouse/hamster/rat antibody production (MAP or HAP or RAP) tests.

††If cells have been grown or cryopreserved in media containing bovine serum, the cells should be tested for the common viral contaminants: bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis (IBR), parainfluenza 3 (PI3), bovine adenovirus, and bovine parvovirus. If human or simian cells are used, it may be appropriate to test these by PCR for bovine polyoma virus.

‡‡Each batch of bovine material used in production should be screened for the absence of bovine viruses or alternatively production control cells can be tested.

§§If cells have been grown using porcine trypsin, they should be tested for the absence of porcine parvovirus and porcine circovirus.

¶¶Each batch of porcine material used in production should be screened for the absence of porcine viruses or alternatively production control cells can be tested.

‖‖‖Cells should be screened for retroviruses using sensitive PCR-based reverse transcriptase assay.

are not genetically modified, further karyology characterization will not be required. At present, regulatory agencies have not approved the manufacture of live human vaccines using cell lines that are tumorigenic at any population doubling used for vaccine production.[24] For some cell lines such as CHO and low-passage Vero and MRC-5 cells, regulatory agencies do not require that the cell is tested for tumorigenic potential. Cell lines that are known to have tumorigenic potential do not have to be tested further. Other less well-characterized cell lines should be tested for tumorigenic potential using an *in vitro* assay such as colony formation in soft agar.[19,24] If the result of the *in vitro* test is negative, an *in vivo* test using a system such as nude mice should be used. Where a tumorigenic cell line has been used for the production of a nonlive vaccine or recombinant protein, the purification process should be validated to show that residual substrate cell DNA has been reduced to less then 10 ng per single human dose. Some manufacturers of adenoviral gene therapy products have also applied this guidance to their purified vector products.

Cells from the MCB and WCB and cells after expansion should be tested for the absence of bacterial and fungal contamination using a sterility assay as defined in EP, US, or Japanese Pharmacopoeias (JP). This sterility testing is applied to the production cells, virus banks, and different stages in preparing the purified vector but is only part of assuring the microbial sterility of the product. Effective control of microbial contamination is shown by environmental, equipment, and operator monitoring during production and also by the validation of the final vial filling process.[20,30,31]

Mycoplasma screening of cell lines should be performed using mycoplasma broth and agar and Vero cell indicator cells as detailed in the EP, JP, or US FDA Points to Consider.[17] Other methods such as PCR can be used for screening cell lines for mycoplasma contamination. However, prior to regulatory acceptance, these newer methods must be validated and shown to detect as wide a range of species and be as sensitive as the classical methods of mycoplasma detection.

Adventitious viral contaminants are detected by the use of several broad specificity screens (*in vitro* virus assay using cell culture, *in vivo* virus assay, and electron microscopy) and *in vitro* tissue culture assays aimed at detecting specific bovine and porcine viruses. Cells should be screened by inoculating production cell lysates onto nonconfluent monolayers of a human diploid cell line (MRC-5), a monkey cell line (Vero), and a cell line of the same species and tissue type as the test cell line. The monolayers should be observed after at least 14 days for the appearance of viral cytopathic effects and hemadsorption. Cell culture supernatants can also be tested for hemagglutinating activity. MRC-5 and Vero cells are susceptible to a wide range of virus species. In order to detect viruses that do not grow or produce cytopathic effects *in vitro*, production cell lysates should be injected into suckling mice and adult mice using a variety of inoculation routes and into the allantoic cavity and yolk sac of embryonated eggs.

The European Medicines Evaluation Agency (EMEA) has stated that wherever possible, the use of bovine and ovine material should be avoided in pharmaceutical manufacture.[23] However, even if producers adapt their virus production methods to serum- or animal protein–free production, the producer cell line will have been cultured or cryopreserved in bovine serum at some point in its history. Producers should comply with the requirements of the guidance to minimize the risk of TSE infection[23] and should screen their cell lines for the absence of contamination by the most common bovine viruses. New draft guidance from the EMEA[32] increases the number of viruses that should be screened for, so that it is similar to US 9CFR recommendations.[33] This guidance also asks for assays to detect neutralizing antibodies against bovine viral diarrhea virus and recommends the use of γ-irradiation to inactivate potential viral contaminants. As porcine parvovirus has been detected in batches of trypsin used in tissue culture, the production cells should also be screened for the absence of this virus.[33]

The presence of endogenous retroviral particles in production cells may be detected by an examination of the cells by transmission electron microscopy. However, the most sensitive methods to detect retrovirus particles are PCR-based reverse transcriptase assays (PBRT). The US FDA has requested that manufacturers of products produced in mammalian or avian cell substrates should utilize this assay to test the cell bank and viral seed.[34] Use of a quantitative PBRT assay on cell culture supernatant will allow the easier differentiation between a positive reaction due to retrovirus-like particles and any reaction due to contaminating cellular DNA polymerase activity.

Testing of Master Virus Bank (MVB) and virus harvests

As indicated above, the testing for adventitious mycoplasma and viral contaminants involves the inoculation of test material onto cultures of indicator cells or injection into live

Table 2 Testing of oncolytic viruses virus used in production

Assay	MVB*	WVB†	Virus harvest	Purified virus
Bacterial and fungal contamination	+	+	+	+
Mycoplasma	+	+	+	–
In vitro virus assay (cpe, HA‡)	+	+	+	–
In vivo virus assay§	+	–	+¶	–
Bovine viruses‖	+	–	+#	–
Porcine viruses**	+	–	+††	–
Retroviruses (PCR-based RTase‡‡)	+	–	+	+¶¶
Endotoxins (LAL‖‖)	–	–	–	+
Host cell DNA	–	–	–	+
Contaminating proteins	–	–	–	+
Virus titration	+	+	+	+

*MVB: Master Virus Bank.

†WVB: Working Virus Bank.

‡Cpe: viral cytopathic effects; HA: hemadsorption.

§*In vivo* virus assay: inoculate neutralized virus into suckling mice, adult mice, and guinea pigs. Test in embryonated eggs if virus is produced in eggs.

¶If virus has been harvested from chicken cells or eggs, then the virus harvest should be tested in embryonated eggs.

‖If virus is produced using cells that have been grown or cryopreserved in media containing bovine serum, the virus should be tested for the common viral contaminants: bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis (IBR), parainfluenza 3 (PI3), bovine adenovirus, and bovine parvovirus. If human or simian cells are used, it may be appropriate to test the virus by PCR for bovine polyoma virus.

#Each batch of bovine material used in production should be screened for the absence of bovine viruses or, alternatively, the virus harvest can be tested.

**If cells have been grown using porcine trypsin, the virus should be tested for the absence of porcine parvovirus and porcine circovirus.

††Each batch of porcine material used in production should be screened for the absence of porcine viruses or, alternatively, virus harvest can be tested.

‡‡Virus should be screened for retroviruses using sensitive PCR-based reverse transcriptase assay.

¶¶If the virus harvest is positive for reverse transcriptase activity, the purified product should be screened to show removal/inactivation of retrovirus-like particles.

‖‖LAL: limulus amebocyte lysate assay for endotoxins.

animals including suckling mice and embryonated eggs (Table 2). To ensure maximal replication of any contaminating viruses, the *in vitro* assay utilize cultures of nonconfluent, dividing cells. Tumor-selective, oncolytic viruses may not replicate in quiescent cells but will multiply in proliferating cells. Where the materials to be tested are virus banks or virus harvests, it is likely that any oncolytic virus or conditionally replicative vector will replicate in the dividing cells or growing animal, resulting in viral cytopathic effects or animal mortality/morbidity. To allow the detection of adventitious viruses, the oncolytic viral vectors will require prior neutralization with specific antibodies. This is an approach that is used during the testing of live human vaccines.[25] The neutralizing antibodies should be of nonhuman, nonsimian origin to avoid cross-reaction with human viruses, or of nonavian origin if the virus has been propagated in avian tissues to avoid cross-reaction with any avian viruses. The antiserum should be prepared using an immunizing antigen produced in cell culture from a species different from that used for production of the vector and free from extraneous agents.

As it may not be possible to document a detailed history of many virus stocks, it is advisable to clone any virus stock by two to three rounds of plaque purification using qualified cells from the production MCB. In this way, potential adventitious viral contaminants of the MVB will be minimized. A MVB that is sufficient to last the lifetime of the product should be produced, using production cells from the MCB or WCB. This MVB should be tested fully using the assays outlined in Table 2 following neutralization where necessary. The testing of subsequent Working Virus Banks (WVBs) and virus harvests can then be less exhaustive. If batches of animal-derived materials used in production are γ-irradiated and tested for possible contaminants, subsequent testing of each virus harvest could be eliminated. If the virus harvest is positive in the PBRT assay, it may be necessary to further characterize the nature of the retrovirus particle in terms of possible infectivity for human cell lines. The retrovirus-like particles from avian cells and Chinese hamster ovary cells have been shown to be defective.[10–12]

In addition to the direct testing of virus seed banks and virus harvests for adventitious contaminants, the testing of production control cells should also be considered. The EP requires that production control cells should be tested during the production of human vaccines and specifies the proportion of each batch of cells that should be tested.[35] The testing of production control cells is relevant when live virus preparations are manufactured in processes with little adventitious virus inactivation/removal capability. Production control cells should be handled where possible in the same way as the cells used for virus growth; they should be grown in the same medium and mock-infected by the same operators. The testing of these control cells is particularly relevant where primary cell lines are used in production or where it is difficult to completely neutralize the virus banks or harvests (see Table 1).

Following harvests from flasks or fermenters, the virus preparations are purified from production cell components and concentrated. Clinical batches of oncolytic viruses have been purified by CsCl gradient ultracentrifugation for phase I clinical trials. Although this method can provide sufficient material for phase I studies, the process is tedious and difficult to scale up. Processes involving column chromatographic methods that are scalable and avoid the use of CsCl have been developed.[36,37]

Purified virus should be tested for bacterial and fungal sterility (bulk-purified virus and vialed material),[38] endotoxin,[39] contaminating host cell DNA,[24] and contaminating proteins.[35] The concentration of the virus should be assessed by titration. If the virus harvest was positive in the PBRT assay, the bulk-purified virus should be screened again in a PBRT assay.[34] Earlier regulatory documents and pharmacopoeial monographs have set a limit of 100 pg of host cell DNA per single human dose, but the most recent EP document[24] on cell substrates for the production of human vaccines increases the DNA content to 10 ng per single human dose. The limit of bovine serum albumin in human vaccines is set to <50 ng per single human dose.[35]

It should be emphasized that the above testing is based largely on that required for human vaccines and viral gene therapy vectors. In the absence of specific recommendations for oncolytic viral vectors, the testing described in this paper will provide a good starting point for decisions in the product development process. To ensure that clinical trials are not delayed, it is recommended that the final package of QC assays is discussed with regulatory authorities.

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