Lot Release and Characterization Testing of Live-Virus–Based Vaccines and Gene Therapy Products, Part 2

Case Studies and Discussion

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The objective of the Well-Characterized Biotechnology Pharmaceutical (WCBP) CMC Strategy Forum is to provide an environment for the development of technical and regulatory consensus positions regarding topics of interest to WCBP. The January 2005 forum was devoted to a discussion of live virus vaccines and viral vectors used for gene therapy. The purpose of that meeting was to determine whether consensus positions could be reached among the delegates regarding lot release, stability, characterization, and comparability testing. The overarching questions posed were

Product Focus: Live-virus vaccines and gene therapy products

Process Focus: Product analysis

Who Should Read: Analytical development, process development and manufacturing, QA/QC, regulatory affairs

Keywords: Characterization, comparability, sterility, purity, safety, potency, identity

Level: Intermediate

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- What is the required testing for lot release and stability of vaccines?
- What is the required testing for lot release and stability of viral vectors for genetic therapy?
- What are the acceptable attributes of a "potency" test?
- What is the best means of quantifying total and infectious viral particles?
- What analytical parameters (obviously including those above) would be considered essential to support "comparability" of a viral product made by a modified process to that of the original process?

In Part 1 (*BioProcess International*, April 2006) we described factors influencing the choices of lot-release and stability assays for vaccines and gene-therapy products. Part 2 presents case studies to illustrate potency testing (for a multivalent vaccine), characterization (for influenza), and comparability studies (for an adenovirus). The article concludes here by summarizing the panel discussion.

Potency Testing of Live Virus Vaccines: A Multivalent Vaccine

The final speaker of the morning was Jim Gombold (Merck & Co., Inc.), who discussed potency testing of live virus vaccines. His case study exemplified issues that can arise during commercial product testing. Commercially licensed viral vaccines

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The ninth Well-Characterized Biotechnology Pharmaceutical (WCBP) Chemistry, Manufacturing, and Controls (CMC) Strategy Forum was held on 9 January 2005 at the Renaissance Mayflower Hotel, Washington, DC. The event was sponsored by the California Separation Science Society (CaSSS; www.casss.org) as part of an ongoing series of discussions between industry and regulatory participants exploring current practices in analytical and bioprocess technologies for development and communication of consensus concepts. The topic of this forum was "Lot Release and Characterization Testing of Live Virus-Based Vaccine and Gene Therapy Products."

Vaccine and gene therapy products based on "live" virus components encompass a wide range of existing and potential medicinal products, with many different potential clinical indications. These viruses may be propagated on a variety of substrates, including eggs and cell culture, and often have a complex composition that must be taken into consideration during testing. The session discussed possible approaches for selecting appropriate lot release, stability, and characterization tests for these products, as well as some unique product-specific challenges such as evaluating the biological activity of live attenuated viruses versus replication-incompetent viruses.

The forum co-chairs were Jim Gombold, Merck & Co. Inc., and Keith Peden, OVRR, CBER, FDA. They were joined on the panel by Mark Schenerman, Denise Gavin, Ziping Wei, Khandan Baradaran, and Anthony Mire-Sluis.

The members of the permanent CMC advisory committee are Siddharth Advant (Diosynth Biotechnology), John Dougherty (Eli Lilly and Company), Rohin Mhatre (Biogen Idec Inc.), Anthony Mire-Sluis (Amgen, Inc.), Wassim Nashabeh (Genentech, Inc.), Nadine Ritter (Biologics Consulting Group, LLC), Mark Schenerman (MedImmune), Heather Simmerman (Amgen, Inc.), Chris Joneckis (CBER-FDA), and Keith Webber (CDER, FDA).

About 45 people attended the day-long forum, representing industry companies, consultant companies, and the FDA.

may contain live-attenuated, inactivated, or recombinant viruses or components of virus subunits.

The choice of assay used to measure vaccine potency depends in large part on the nature of the product. Live-attenuated vaccines require biological assays, such as plaque assays and $TCID_{50}$ assays, because infectivity is thought to be the primary factor contributing to their potency. In contrast, noninfectious vaccines (inactivated or virus subunit vaccines) generally rely on assays such as enzyme immunoassays that measure structural virus components. Vaccines based on recombinant viral vectors are infectious, although usually replication defective, and potency assays for such products may focus on expression of a transgene-encoded protein rather than infectivity of a vector.

Potency testing of multivalent products is generally more complicated than the testing of monovalent products. Assays may require modifications to ensure specificity, such as when other viral components must be neutralized. Component viruses may interact, either interfering with or augmenting viral replication. Because of these concerns, reference standards used in calibrating assay results must be carefully chosen. Heterologous standards (those that do not directly reflect product composition) may result in misleading interpretations of the data if they do not behave identically to the product in a chosen assay.

To illustrate issues that can be encountered during potency testing of live virus vaccines, a case study focused on a quadrivalent measles, mumps, rubella, and varicella (MMRV) vaccine. Because the vaccine components are live-attenuated viruses, biological infectivity assays are used to measure potency for both release and stability testing. Both virus–virus and virus–matrix interactions occur that can, under some circumstances, lead to erroneous potency measurements. Specific assay issues encountered include the need for neutralizing antisera for assay specificity, the occurrence of sample dilution bias (which complicates calculation of final potencies), and the impact of quadrivalent (homologous) compared with monovalent (heterologous) reference standards.

Virus Interactions: Routine testing of vaccines involves antiserum-mediated neutralization of three of the four components in MMRV before actual sample titration. This is necessary to prevent false positive results such as mumps-induced CPE during potency testing of measles in a $TCID_{50}$ assay. Neutralization has the added benefit of reducing or preventing unwanted interactions between viruses in multivalent products.

Disclaimer

It must be noted that the details contained in this manuscript reflect the discussion that occurred during the January 2005 CMC Strategy Forum, in addition to the personal experiences of the authors. However, this document does not represent officially sanctioned FDA policy or opinions and should not be used in lieu of published FDA guidance and points to consider or direct discussions with the agency.

Some assays, such as those based on PCR (polymerase chain reaction) detection, are inherently specific and do not necessarily require neutralization to impart specificity. Consequently, all viruses in the product undergo simultaneous replication. Replication of one virus may therefore inhibit or augment replication of another.

Such interactions have been observed with the MMRV vaccine using PCR-based approaches. They result in changes in relative levels of viral nucleic acids in cells infected with all four viruses versus those infected with a single virus (with the other three viruses present but neutralized). For example, in MMRV, interaction between two viruses results in a 30% reduction in genome level for one virus with a simultaneous 2- to 3-fold increase in genome level for the other. The degree of suppression is

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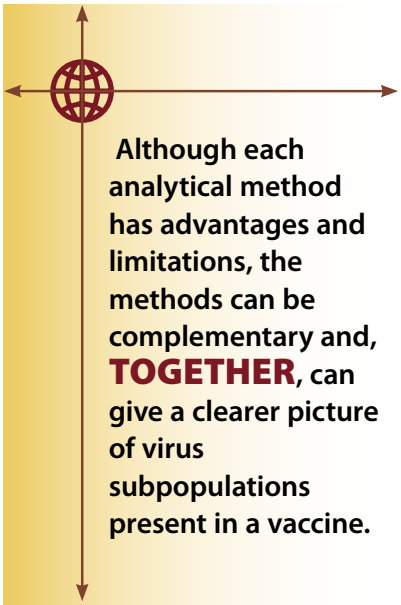

generally small, usually observed only in more sensitive assays, and depends on the relative amounts of the two viruses.

Interactions between a virus and the vaccine matrix (sum of all components in the vaccine) can also affect potency of a combination live-virus vaccine. This is more of a process issue than an assay issue. As an example, in MMRV vaccine preparation, four monovalent bulks are mixed in proportions necessary to achieve a target potency for each component in the final product. Analytical studies demonstrate that such mixing can lead to a rapid and irreversible loss of potency for some viruses. Potency assays must be sufficiently accurate and precise to measure that loss so that appropriate process changes can be implemented and the desired viral potency achieved.

Assay Considerations: Due to the multiinfectious nature of the MMRV product, potency testing requires procedures to selectively neutralize individual viral components to achieve accurate results. A benefit of this neutralization approach is that it prevents or mitigates virus–virus interactions that could otherwise lead to inaccurate results.

However, incorporating the neutralization step complicates the assay by requiring additional characterized and qualified reagents. Neutralization procedures lengthen overall assay test times because of additional time needed to set up and incubate neutralization reactions. In addition, some antisera used in varicella potency testing of the MMRV vaccine produced a nonspecific inhibition of varicella replication in the assay (not due to a specific antivaricella response). Although antiserum reagents are specifically qualified to assure that such attributes are within an acceptable range, using a homologous reference standard was critical to producing accurate results for the varicella potency of MMRV lots (see discussion below).

Assay dilution bias is a common problem for quantitative analytical methods, typically attributable to inherent properties of samples being analyzed. Some dilution bias was observed in the varicella potency assay. In this assay, virus is serially diluted before inoculation onto cells. The number of plaques that form is expected to be inversely proportional to dilution of the sample. Theoretically, the dilution-corrected potencies (observed potency times dilution factor) for all dilutions should be identical. However, for varicella potency testing of MMRV, the dilution-corrected potency continually increases with greater sample dilution. The magnitude of bias can vary from 0% to 20%, which may require mathematical adjustment of data to ensure meaningful potency results. Experience has shown that different bulk manufacturing processes and different formulations can exhibit different degrees of dilution bias.

Potency assays are typically calibrated against well-characterized reference standards to ensure testing consistency from day to day within a test lab and uniformity in testing between labs. The realities of testing a multivalent product such as MMRV can also affect the choice of reference standard used for calibration. In the simplest sense, the standard may be monovalent or multivalent; in the latter case it is likely to be a product lot made specifically for that use.

In Summary: Standards that are not identically formulated and similar in potency to a test article may be considered heterologous and, as such, may not be the best choice for calibration. A monovalent standard may not be treated in an assay the same as the product (e.g., subjected to neutralization to ensure testing specificity) and may therefore behave differently from the sample. Thus, using homologous reference standards minimizes the potential for complex interactions and biases that could cause artifacts.

Characterization of Virus Subpopulations: Influenza

Ziping Wei (MedImmune) presented an overview of techniques used to characterize virus subpopulations. Influenza virus preparations can contain a heterogeneous mixture of infectious and noninfectious viral particles with varying states of aggregation, particle counts, and morphology. Characterization of such subpopulations is important for viral vaccine manufacture. Understanding these parameters and their correlations can assist in development of a robust process and a homogenous product. For example, the ratio of infectious particles to total particles is a parameter that can be used to characterize virus preparations. However, it remains unknown whether those differences have any impact on vaccine performance, so interpretations of the results must be carefully assessed.

Several techniques can be used to assess total virus particle counts, size heterogeneity, morphology, and potency (Table 1). These include field flow fractionation (FFF) followed by multiangle light scattering (MALS), transmission electron microscopy (TEM), atomic force microscope (AFM), quantitative polymerase chain reaction (Q-PCR), density gradient centrifugation (disc centrifuge or analytical ultracentrifugation), infectivity assays ($TCID_{50}$ or plaque assays), and fluorescent focus assays (FFA).

FFF–MALS is a relatively new technique for measuring virus particle size, size distribution, and total particle counts. It does not rely on any

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standard for particle size and count determination, offers good precision and reproducibility, and provides high-throughput with low-cost. Method accuracy was confirmed by analyzing polystyrene beads and adenovirus samples with known sizes and concentrations. The main limitation of this technique is that it depends on good FFF separation for an accurate measurement.

TEM is an established and widely used technique to assess virus particle size, morphology, and counts. Although it is the "gold standard" in the field and provides the advantage of sample visualization, it is costly, has low-throughput, and may cause some artifacts due to heavy-metal staining of samples. In terms of quantitation, TEM has low precision and relies on bead standards. AFM is similar to TEM and can be used for characterizing virus particle size, morphology, and counts. Sample preparation induces fewer artifacts than TEM but poorer resolution. PCR measures a specific gene copy and is an indirect way to determine total particle counts. It has low cost, high throughput, and is quantitative; however, nonparticle–associated DNA or RNA may lead to artificially high copy numbers, and empty capsids are not included in total particle counts.

Potency assays such as plaque and $TCID_{50}$ assays, which measure the ability of virus to infect cells, typically take several days to complete, but are classic methods for determining virus infectivity. FFA, using fluorescent cell-counting procedures, is often a faster potency assay. Although each analytical method has advantages and limitations, the methods can be complementary and, together, can give a clearer picture of virus subpopulations present in a vaccine. Choice of a specific method(s) depends on the vaccine characteristics being evaluated.

Several case studies for influenza virus were presented. Influenza virus is polymorphic, with varying size and shape. The first case study showed correlation between total particle counts, infectivity, and size distribution of four B-strain influenza virus samples determined by $TCID_{50}$, TEM, FFF–MALS, and PCR. TEM, FFF–MALS, and PCR provided comparable data on total particle counts. The percent infectious particle values were determined from the ratio of infectivity, as determined by $TCID_{50}$ to total particle counts, which were determined by either TEM or FFF–MALS. The values determined by either method were comparable. Although the samples had a significant level of aggregates (as determined by FFF–MALS and confirmed by TEM), there were insufficient data to determine whether that aggregation affected potency of the virus samples.

The consensus was that it is good to determine a correlation between aggregation and potency in in vitro assays. SAFETY concerns were raised about aggregates that may cause UNWANTED immunogenicity, such as for gene therapy products.

A second case study used FFF–MALS and FFA methods to measure polydispersity and total particle count of type A and B influenza samples before and after sample processing and excipient addition. The results demonstrated that sample treatment caused disaggregation and that an increase in total particle counts was due to sample concentration. This sample treatment produced more homogenous products for virus size distribution regardless of the virus type and initial sample aggregation state.

A third case study described the effect of temperature on total virus particle counts and size distribution of two type B virus samples by FFF–MALS. Upon warming to 33 °C for 30 or 90 minutes, the samples showed no significant changes for total particle counts (the number of virus particles per unit volume, including the individual virion within each aggregate), but the level of aggregation was dramatically reduced. These results indicate that certain aggregate populations were reversible and could be converted to monomers by changes in temperature. In summary, FFF–MALS provided an efficient way to monitor transitional changes in virus size distribution caused by experimental treatments.

Comparability of Gene Therapy Products: An Adenovirus

Khandan Baradaran (Biogen Idec) presented a discussion on comparability. Comparability studies are required when manufacturing changes are made. Such changes include, but are not limited to, changes in cell line or virus vector/seed, manufacturing site, process, formulation, and container. Testing depends on the product, the process change, and whether that change occurs during clinical trials or postlicensure. Potency and safety testing are important, but other tests used to characterize a product, such as process or product residuals tests and additional stability assessments, are also good indicators of comparability.

At Biogen Idec, cell-culture scale, order of process steps, and final product vial were changed during early clinical trials of a recombinant adenovirus gene delivery product. Release testing evaluated appearance, pH, particle concentration, infectivity, transgene activity, replication competent adenovirus, residual host cell DNA, impurities by SDS-PAGE, sterility, and endotoxin. Testing for adventitious agents, mycoplasma, AAV, and bioburden is performed on the cell culture harvest. Characterization included testing for residual medium and process components (such as BSA, benzonase,

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Table 1: Bulk and final container characterization assays

Assay	Purpose	References/ Comments
Field flow fractionation multiangle light scattering (FFF–MALS)	Determine particle number and aggregation state	**17, 18**
Atomic force microscopy (AFM)	Determine particle number and aggregation state	**19, 20**
Transmission electron microscopy (TEM)	Determine particle number	**21, 22**
Size exclusion chromatography multi-angle light scattering (SEC-MALS)	Determine particle number and aggregation state	**23**[a]
TCID, FFA, plaque, or other assays	Determine proportion of defective particles based on the difference between total particles and infectious particles	**24–26**[b]
Polymerase chain reaction (PCR)	Determine proportion of nucleic acid containing particles	**27**
Density gradient centrifugation	Determine proportion of defective particles based on relative densities of particle populations	**28, 29**
Analytical ultracentrifugation (AUC)	Determine proportion of defective and aggregated particles based on hydrodynamic properties of particle populations	**30**
Capillary electrophoresis (CE)	Determine proportion of defective and aggregated particles based on particle mass and charge	**31**
Reversed-phase HPLC (RPHPLC)	Determine proportion of defective and aggregated particles based on hydrophobic interaction properties	**32**
Ion-exchange chromatography (IEC)	Determine proportion of defective and aggregated particles based on charge state of the particles	**33**
Size exclusion chromatography (SEC)	Determine proportion of defective and aggregated particles based on hydrodynamic sieving properties of particle populations	**34**
SDS-PAGE (or equivalent)	Determine composition of proteins contained in preparation based on polypeptide chain sizes	**35**
Western blot	Determine composition of immunoreactive proteins contained in preparation	**36**
Process residuals (BSA, benzonase, polysorbate, etc.)	Quantify process-related impurities	**5, 6**[c]

[a] May not separate large aggregates due to upper exclusion limit of SEC

[b] May not be relevant to all viruses

[c] May be a release assay depending on process and stage of clinical development

polysorbate) and DNA structure by restriction analysis. Additional characterization testing included analytical ultracentrifugation (AUC) analysis of purified drug product to measure virus subpopulations and aggregates of adenovirus particles.

Critical information about aggregation, empty capsids, virus subparticles, and other lower molecular weight species was gained from AUC experiments. Through boundary sedimentation velocity experiments and Sedfit computer analysis (a data analysis software for sedimentation velocity, sedimentation equilibrium, and dynamic light scattering) (**38**), monomeric virus particle concentration can be determined based on the change in refractive index. The aggregates, empty capsids, and low molecular weight degradation products can also be quantified. Small volumes of sample (as little as 100 µL) can be measured directly in the formulation buffer. This information can be used to monitor structural heterogeneity of different batches and demonstrate manufacturing consistency.

Bridging stability studies were also conducted. Test results from product made by subsequent processes were compared with existing reference standards manufactured by the original process. In all cases, comparability was demonstrated and changes were reported as amendments to existing INDs.

Table 1 lists a number of tests for characterization and comparability studies of viral products. Together they give a comprehensive picture of virus subpopulations including viral particle size, size distribution, potency, and impurities. The choice of specific methods for a comparability protocol depends on the type of product and the relevance of information to its efficacy and safety.

Participants agreed during the afternoon panel discussions that a comparison between test results from multiple methods is useful in making correlations between the physicochemical properties of virus subpopulations and their activity (e.g., percent aggregates versus percent infectious particles). The consensus was that it is good to determine a correlation between aggregation and potency in in vitro assays. The question was raised whether such a correlation is relevant in vivo. Safety concerns were raised about aggregates that may cause unwanted immunogenicity, such as for gene therapy products.

Participants agreed that methods used to assess comparability vary by product and the nature of manufacturing changes. Changes such as those to virus seed, scale, site or resin, and container and formulation would require comparability assessments. The need for further clinical studies is determined by the manufacturing change and amount of information available for a product and process. The ability to measure efficacy, safety, and quality through in vitro assays can determine the extent that further toxicological or clinical studies are needed. The route of administration and bioavailability should be considered when formulation changes are made.

Characterization tests that should be used to demonstrate comparability

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were recommended to include robust and relevant technologies, potency, and physicochemical methods. It was recommended that the scientific rationale for such tests make sense with regard to a proposed manufacturing change. Test methods can also depend on the purity of a virus preparation. Ion-exchange HPLC was mentioned as a common and robust technique. There was general consensus that potency assays are important in comparability assessments.

The need for additional stability studies in comparability assessments depends on whether a particular manufacturing change is likely to affect stability. Formulation and container changes usually require additional stability studies. Although both real-time and accelerated stability studies are useful, it was pointed out that accelerated stability studies are useful even for minor changes. There was general consensus that when a manufacturing change affects drug substance, both drug substance and drug product should be evaluated for stability changes. The question was raised whether a more stable product following a manufacturing change is desirable or not.

Panel Discussions

The sessions were followed by three panel discussions of issues raised in the presentations. The first panel covered topics from the first three talks. One point was that FDA requirements for a viral-vectored vaccine differ from those for the same viral vector when it is used in gene therapy. Concern was also expressed that identity testing, potency testing, and limits for residual DNA and protein differed depending on a product's intended indication and where that product is reviewed. Some participants thought that requirements for gene therapy products were more stringent than those for vaccines. The allowable level of host proteins was discussed, because OVRR and OCTGT appear to have different views about whether proteins present a risk. Keith Peden mentioned that because proteins cannot replicate themselves and thus will be rapidly diluted, proteins have not been considered by OVRR to be a safety concern. For manufacturing consistency, however, the amount of protein needs to be specified for vaccines and gene therapy products.

The issue of residual host DNA is specifically important for tumorigenic cells. Partipants discussed the limits of DNA from different types of cells and whether it made sense to set limits. In addition, because different viral vectors cannot be purified to the same extent as others, the route of inoculation may influence the limits, and the DNA size may be of concern. Dr. Peden explained that the WHO limit of less than 10 ng DNA per dose for vaccines manufactured in all continuous cell lines (whether tumorigenic or not) was not accepted by the FDA because it is unclear that DNA from highly tumorigenic cells has the same oncogenic potential as DNA from nontumorigenic cells. Experiments are under way at CBER to address this issue. There was discussion about whether size reduction would lessen the potential risk. Dr. Peden indicated that OVRR considers that clearance of DNA (reduction in amount and inactivation) should be validated.

The suitability of various potency assays and whether the two offices have different requirements was discussed, including the issue of whether in vitro assays were acceptable or even preferred in some cases. There was consensus that in vitro expression assays and infectivity assays, such as plaque assays, can be used to measure potency. However, Denise Gavin pointed out that infectivity assays have not been accepted by OCTGT as potency assays for gene therapy products; a biological assay relevant to the product is required. In conclusion, participants agreed that the two offices recognized the difference in requirements for release of vaccines and gene therapy products and would work to harmonize those requirements where appropriate.

The afternoon session comprised presentations and panel discussions on characterization of virus subpopulations and comparability studies following manufacturing changes. Virus subpopulations were defined as aggregates, immature or defective particles, and genetic variants. Several techniques used to characterize virus preparations were presented. These techniques are designed to assess particle count, size heterogeneity, morphology, and potency. The techniques discussed included FFF–MALS, TEM, Q-PCR, density gradient centrifugation, plaque assays, and FFA. FFF–MALS is being used to assess total particle counts and size distribution. The analytical ultracentrifugation technique appeared to be promising for measuring total monomeric particle counts, aggregation levels, and virus subpopulations such as assembly intermediates and degradation products.

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