An initiative to manufacture and characterize baculovirus reference material

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Letter to the Editor

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ABSTRACT

This letter to the editor brings to the attention of researchers an initiative to develop a baculovirus reference material repository. To be successful this initiative needs the support of a broad panel of researchers working with baculovirus vectors for recombinant protein production and gene delivery for either therapy or vaccination. First there is a need to reach a consensus on the nature of the reference material, the production protocols and the baculovirus characterization methods. It will also be important to define repository and distribution procedures so that the reference material is available to any researcher for calibrating experimental data and to compare experiments performed in the various laboratories. As more and more baculovirus-based products are licensed or in the final stages of development, the development of a repository of baculovirus reference material is timely. This letter describes the requirements for the reference material and for the project as a whole to be successful and calls for a partnership that would involve academic, industrial laboratories and governmental organizations to support this international initiative.

1. Background

In the last decade, the baculovirus/insect cell technology platform, often referred to as the Baculovirus Expression Vector System (BEVS), has been used to manufacture vaccines that are licensed or are in the late phase of approval. Building on the high safety profile of baculovirus vectors and the high production yield of insect cells, which are two important criteria for vaccine manufacturing (biosafety and cost effectiveness), researchers have developed numerous sub-unit vaccines and vectored vaccines that have been proven to be efficient (Mena and Kamen, 2011; van Oers, 2006). For example, GlaxoSmithKline's Cervarix™ (GSK, Rixensart, Belgium), a bivalent human papillomavirus VLP vaccine against cervical cancer, was approved by the European Medicines Agency in September 2007 and the United States Food and Drug Administration (US FDA) in October 2009. Another FDA approved baculovirus product is PROVENGE®, an autologous cellular immunotherapy for the treatment of prostate cancer (Kantoff et al., 2010). Candidate human vaccines that are in late-stage clinical development include FluBlok®, a recombinant haemagglutinin-based trivalent seasonal flu vaccine developed by Protein Sciences Corporation (PSC, Meriden, Connecticut, USA) (see Cox and Hashimoto, 2011), and Dyamid®, a therapeutic vaccine for the treatment of type 1 diabetes mellitus, developed by Diamyd Medical AB (Stockholm, Sweden). Baculovirus-based veterinary vaccines preceded the human vaccines and the first of these veterinary vaccines on the market was Porcilis Pestis® (Intervet/Schering-Plough, the Netherlands) directed against Classical swine fever in pigs. It was followed by various other veterinary vaccines, such as Ingelvac® CircoFLEX (Boehringer Ingelheim Vetmedia Inc., USA) or Porcilis® PCV (Intervet/Schering-Plough, the Netherlands), against Porcine Circovirus type 2. The marketing authorization by regulatory agencies in Europe and North America for these products has thus paved the way for licensing of many other BEVS-derived veterinary vaccines that are currently under development. These examples clearly indicate that the BEVS and insect cell technology have reached the stage of a robust manufacturing platform meeting all of the regulatory requirements to register novel biologics.

Furthermore, since the discovery in the mid 1990s by Hofmann et al., (1995) of efficient transduction of hepatocytes using baculovirus vectors, a broader use of BEVS has been made in gene transfer experiments. This has led to the expression of foreign proteins in mammalian cells (Kost et al., 2005) and to the development of baculovirus vectors to successfully deliver transgenes in animal models for instance for gene therapy or vaccination purposes (Hu, 2010; Madhan et al., 2010).

Thus, recombinant baculovirus vectors are increasingly being used in the production of new biologics and as a new gene delivery system; the latter so far mainly in preclinical studies. In spite of the extensive use of the BEVS in manufacturing commercial vaccines, the baculovirus system still lacks standardization. The absence of baculovirus reference standards impedes comparison of titers and therefore effectiveness of baculovirus vectors made in different laboratories. In fact, although many manufacturing techniques are available, all of which allow reproducible results within a single setting, the characteristics of baculovirus vector batches cannot be compared from one laboratory to another. Recently, Roldao et al. (2009) performed a comprehensive analysis of different techniques in order to evaluate their merits and drawbacks giving an intra-laboratory perspective on how to compare various titration methods. Still, there is no clear conclusion as to which method is the best and, in addition, new methods are continuously being developed (Ferris et al., 2010; Transfiguracion et al., 2010). Most

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methods for baculovirus titration do not necessarily give the number of virus particles, but they are rather a measure proportional to the concentration of infectious virions present in the batch. It has been shown that titers for the same viral preparation, even with shared protocols, will significantly vary from one laboratory to another (Lock et al., 2010), unless these laboratories can standardize/normalize their assay based on a set value determined against well characterized common reference materials. This has been highlighted as a significant issue when trying to compare, for example, various systems used to produce baculovirus vectors for gene therapy (Aucoin et al., 2008).

One way to address the issue of precision, and hence improve inter-laboratory comparisons, is to make a well characterized baculovirus reference material available to all researchers. It is recognized that in order to gain acceptance, the reference material has to result from an international, coordinated effort made together by academic and industry researchers as well as regulatory body representatives, who have a vested interest in the success of baculovirus-based technologies. It should be recognized that the baculovirus reference material (BRM) does not need to meet all of the requirements defined in the current good manufacturing practice (cGMP) guidelines. However, elements of the good manufacturing practice will require characterization and documentation of the BRM in such a way that it can be used to calibrate internal standards and to normalize analytical methods used by individual laboratories.

The idea of a standard viral reference material is not novel, and this initiative follows two other major initiatives hosted by the International Society for BioProcess Technology (http://www.isbiotech.org/refmaterials.html) initiated by the Adenovirus Reference Material Working Group (ARMWG) and the Adeno-Associated Virus Reference Material Working Group (AAVRMWG) (Hutchins et al., 2000; Lock et al., 2010). Contrary to adenoviruses, adeno-associated viruses, or lentiviruses, which are more widely used in clinical gene therapy trials, it is generally recognized that baculovirus vectors for human gene therapy are still in their infancy. There is, however, a significant body of knowledge that can be used to shape and help advance the use of baculovirus vectors in this area and in vaccine technology.

During the Advanced Symposium on Viral Vectors for Gene Therapy in Kuopio, Finland, August 26-September 4, 2010 (http://www.kuopioembolabcourse.easco.org/) a formal discussion was held on creating a Baculovirus Reference Material Working Group (BRMWG) that would combine efforts from academia, industry and governmental agencies to achieve a well characterized BRM within reasonable time lines. After a series of short presentations on baculovirus molecular design, insect cell culture and modes of production, downstream processing, and a review of quantification and characterization methods, the panel engaged in a discussion to shape the approach for developing the BRM. The key points of this panel discussion are summarized in the following sections.

2. Choice of the baculovirus

Similar to the AAV reference material already developed, it has been suggested that 1) the most common baculovirus used as vector will be chosen for the reference material; and 2) the baculovirus should be in its vector form and not wild-type. The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the obvious choice for the Baculovirus Reference Material Working Group (BRMWG) to satisfy the first criterion. Apart from being used most commonly, it is the type member of the genus Alphabaculovirus (Jehle et al., 2006) and its genome was the first to be completely sequenced (Ayres et al., 1994). The second criterion, however, requires some discussion. Vectors derived from two strains, C6 and E2, have been suggested as candidate reference material. These two strains are clonal isolates from a virus, which was originally isolated by Vail and colleagues from a single insect larva (Vail et al., 1971) as reviewed in (Cohen et al., 2009). These clonal isolates have been developed into different baculovirus vectors. Bac-to-Bac (Invitrogen) is derived from the E2 strain while BaculoGold (BD Biosciences), BacMagic (Roche/EMD) and flashBAC (Oxford Expression Technologies) are derived from the C6 isolate (see van Oers, 2011). The Bac-to-Bac system may be ideal for storage purposes, as one would rather maintain bacmid DNA (Luckow et al., 1993) as a "Master" element for BRM. It should be clear that the budded virus (BV) form of the virus is the form to be included in the BRM as that is the form used for AcMNPV vectors in cell culture.

A lot is known about baculovirus genes and their function in the infection process (Cohen et al., 2009; Rohrmann, 2008), but there is no direct evidence that variation in specific genetic regions between the E2 and C6 isolate results in differences in activity of the derived vectors. Therefore, it may not be seen as a priority from a user standpoint, to add complete sequencing of the vector genome to the list of characterization necessary for the BRM. The BRM, however, will be a material that will be kept for an extended period so characterization is needed to check genetic stability over time. In addition, sequence comparison with vectors used in a particular setting is likely to be required. The BRM material may be used by individual researchers as template to amplify baculovirus genes, and hence the sequence of the starting material is important as well.

The vector to be constructed should include a reporter gene for assessment of the quality of the vector. Given that baculovirus vectors are considered for use in both mammalian and insect cell systems, a reference material that has a fluorescent reporter gene downstream of both a mammalian cell/viral promoter and a baculovirus promoter has been suggested. A few examples already exist of baculovirus vectors with a green fluorescent protein (*gfp*) gene downstream of both a baculovirus p10 or polyhedrin (*polh*) and a CMV promoter (Hu et al., 2003a,b; Urabe et al., 2002). The use of green fluorescent protein also maintains consistency with other viral reference materials (e.g. adeno-associated virus, AAV).

3. Choice of cell line

The cell line used for producing the BRM should preferably be the same as the cell line used for titrating the infectious nature of the baculovirus. As with all enveloped viruses, part of the character of the baculovirus is provided by the cell line from which it is derived; therefore, for consistency, the production cell line should be a cell line that is accessible to all laboratories. The cell line of choice for the production of the BRM should allow for production of large quantities of infectious baculovirus vectors, preferably in serum-free medium and in suspension cultures. The latter is preferred for ease of culture in the various laboratories and for insect cell bioreactor systems.

Of the candidate cell lines, the Sf-9 line (Vaughn et al., 1977) is the most likely candidate for the generation of the BRM, in terms of accessibility and ease of manipulation. Sf-9 cells are still the most commonly used cell line for both vector stock generation and production of recombinant protein, and may also be better at generating budded baculoviruses (Aucoin et al., 2010). The mode of operation for the production of the virus, as long as it is standardized, appears to be less important than the characterization of the final product. The choice of Sf9 cells would also allow the production of baculovirus at higher cell densities than what is achieved with other cell lines.

4. Composition, preparation and storage of the BRM

As the aim of the BRM is to serve as a benchmark material for researchers attempting to develop these vectors for different purposes, the reference material should be free of contaminants from the cell culture in which it was produced. The most important criterion here appears to be the absence of residual protease activity, because this is the most important contamination, which might lead to inactivation/degradation. The degree of purity of the material, however, brings into question how stable the BRM will be. The longest storage stability studies have looked at a period of ~300 days (Jorio et al., 2006). Ion exchange chromatography has been shown to yield the best results so far for the purification of baculovirus. Cation-exchange materials have the best reported features, being able to capture virus directly from the supernatant and elute at relatively low salt concentrations, as well as yield the greatest amount of active virus (Vicente et al., 2009; Wu et al., 2007). The purity of the final material is best shown as a ratio of genomes to protein. In contrast to smaller and simpler non-enveloped viruses, SDS–PAGE of the virus does not give a sufficient defining pattern, although certain proteins are generally recognizable including the viral envelope protein GP64 and the capsid protein VP39 (Hu et al., 2003a,b; Transfiguracion et al., 2007).

Fresh medium is said to have many cryopreservant features, although short term stability studies have shown that purified baculovirus in PBS in the presence of a known cryopreservation agent such as dimethyl sulfoxide (DMSO), glycerol or sucrose can also retain baculovirus activity (Jorio et al., 2006). The latter may be the best and most widely accepted for a BRM, and has been shown to maintain the activity of the virus when stored at −80 °C or in liquid nitrogen. On-going work to evaluate the potential for baculovirus lyophilisation may lead to an alternate approach for the long term storage of baculovirus, but to date, there is no published report to support this approach.

5. What aspects of the baculovirus reference material should be included?

Similar to other viruses used as gene delivery vectors and consistent with prior knowledge in the field of baculovirology, a sample preparation of virus may be composed of several populations. Most simply, there is often a population of functional "bioactive" particles, as well as subpopulations of defective particles. In this field, there has been significant work trying to understand how these various populations are generated and propagated (De Gooijer et al., 1992; Kool et al., 1991; Krell, 1996; Pijlman et al., 2002; Pijlman et al., 2003). Hence, two types of characterizations are suggested: a functional characterization (see below) and a physical characterization that measures the total number of physical viral entities. Consequently, at least two consistent measures to quantify and qualify a virus sample are needed. This type of characterization is common for gene therapy applications where vectors are assessed for their efficiency of transduction or infection and characterized to determine the total number of viral particles per dose to control the total viral load eventually injected into a patient. The ratio of functional to total virus particles is an important quality attribute of the final viral preparation. However, this ratio is highly dependent on the quantification methods used and remains to be defined for baculovirus vectors.

6. Functional titers

The cell line and medium that will be used to judge the "bioactivity" of the virus in terms of infectious and transducing units are an important aspect of assay standardization. These cell lines should be available through the existing repositories, and, as stated previously, should be consistent with the production cell line (Sf9). Both infectious and transducing titers will need to be evaluated before and after storage in order to evaluate the eventual loss in functional titer due to storage in the repository. These data will dictate the volume of material that should be prepared and the "shelf-life" of the BRM.

6.1. Infectious titer

The infectious titer should relay the ability of the virus to replicate in insect cells. Commonly used methods include plaque assay, end-point dilution assay to determine the Tissue culture infective dose 50 (TCID50) and variations thereof (King and Possee, 1992; Lynn, 1992; Mena et al., 2003; O'Reilly et al., 1992; Pouliquen et al., 2006). The BRMWG is recommending the incorporation of an end-point dilution assay based on the detection of green fluorescent protein. It will be important to specify the medium and incubation conditions that are used to obtain the infectious titer reported for the BRM.

6.2. Transducing titer

The transducing titer should relay the general ability of the vector to transduce mammalian cells. The CMV promoter is a strong promoter that is active in many mammalian cell lines such as HeLa or HEK293 cells (Chan et al., 2006). Also in this case, it will be important to specify the target cells, the medium and the incubation conditions that are used to obtain the transducing titer reported for the BRM.

7. Physical titers

The total number of physical particles often differs from the number of infectious particles detected. Methods based on the detection of the number of genomes or detection of complete particles have often been used to gain an appreciation of the total particle count. Viral genome copies, directly reflecting the number of budded virus particles, could be assessed using real time-PCR (Hitchman et al., 2007; Lo and Chao, 2004; Zwart et al., 2008). Transmission electron microscopy (TEM) and flow cytometry (FCM) are two other methods that could be used for determining the total particle count (Shen et al., 2002). Given the potentially restricted availability of TEMs, quantitative PCR and FCM are the likely candidates for detailed characterization of the physical titers of the BRM.

8. Concluding remarks

Because of the well-defined safety profile of the baculovirus expression system, the well-established high titer production methods, the availability of a panel of analytical methods for quantification, and the increasing number of approved applications the time is right for the design of baculovirus reference material. The overall goal of the BRM project is to reach a consensus about the nature and production method of the BRM material supported by a sufficient number of laboratories that will be engaged voluntarily in this project. So far, preliminary discussions with representatives from companies, universities and governmental organizations were very positive and confirmed strong support for the initiative. Very important to a BRM is the availability of well-characterized and documented materials and protocols for characterization of individual BRM batches. These protocols should be general enough to allow any laboratory wishing to standardize their work and to conduct similar assays for comparison and benchmarking. Furthermore, it is critical that the material generated as reference standard is stable while stored in a general

repository so that the properties determined before storage are the same once the material is thawed. Adequate financial resources are crucial to the success of this initiative as well as the cooperation of the users. Users will need to participate in the testing phase to help develop the reference material and for later use of the BRM to standardize their work.

For more information and to submit comments on this project, please go to http://www.isbiotech.org/ReferenceMaterials/baculo-ref-home.html.

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Conflicts of Interest

There are no conflicts of interest for Amine A. Kamen, Marc G. Aucoin, Otto-Wilhelm Merten, Kari Airenne, Yu-Chen Hu, Mauro Mezzina, and Monique M. van Oers. Paula Alves works for IBET, Portugal. Yoshifumi Hashimoto is employed by Protein Sciences Corporation.

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