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Minireview

Diagnostic approaches for viruses and prions in stem cell banks

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Abstract

Some stem cell lines may contain an endogenous virus or can be contaminated with exogenous viruses (even of animal origin) and may secrete viral particles or express viral antigens on their surface. Moreover, certain biotechnological products (e.g. bovine fetal serum, murine feeder cells) may contain prion particles. Viral and prion contamination of cell cultures and "feeder" cells, which is a common risk in all biotechnological products derived from the cell lines, is the most challenging and potentially serious outcome to address, due to the difficulty involved in virus and prion detection and the potential to cause serious disease in recipients of these cell products.

Stem cell banks should introduce adequate quality assurance programs like the microbiological control program and can provide researchers with valuable support in the standardization and safety of procedures and protocols used for the viral and prion testing and in validation programs to assure the quality and safety of the cells.

Keywords: Stem cell banks; Endogenous viruses; Exogenous viruses; Prion particle; Cell therapy; Diagnostic methods

Introduction

The new treatments of cell therapy based on the transplant of cells of human origin still have problems, such as the difficulties of culture and differentiation of the cells, the possibility of chromosomal alterations and potential for tumorigenicity. Of particular importance is the possibility of transmission of infectious diseases to the recipients of these cell products. Any microbial contamination of the donor's biological products or introduced during manufacturing process can potentially present a serious hazard to recipients even if it is not an overt pathogen. The most common potential forms of contamination (e.g. bacteria (include mycoplasma), yeast, fungi) can be readily assessed on a routine basis (European Pharmacopeia, 2004a, 2004b; Cobo et al., 2005). However, viral contamination of cell cultures and feeder cells, which is a common risk in all biotechnological products derived from the cell lines, is the most challenging and potentially serious outcome to address, due to the difficulty involved in virus detection and the potential to cause serious disease in recipients of these cell products (Cobo et al., 2005).

To ensure the provision of safe and reliable cells and tissues for these applications, it is necessary to regulate the procurement, processing, testing, preservation, storage and distribution of all cells that will apply in the human body (Directive 2004/23/CE). Stem cell banks must assure the quality and safety of these cells, and these aims are particularly important in the avoidance of transmissible diseases like viral and prion infections that are difficult to diagnose. These establishments should introduce adequate quality assurance programs like the microbiological control program. In this respect, accredited stem cell banks can provide researchers with valuable support in the standardization and safety of procedures and protocols used for viral and prion testing and in validation programs to assure the quality and safety of the cells.

This review will discuss the methodology that should be used in the stem cell banks in order to assure the quality of cell and biotechnological products and avoid the transmission of infections, in particular those that involve virus and prion particles.

Viral and prion contamination sources of stem cell lines

Some lines or cell cultures may contain an endogenous virus or can be contaminated with exogenous viruses, and may secrete viral particles or express viral antigens on their surface.

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Moreover, certain biotechnological products (e.g. bovine fetal serum) may contain prionic particles. The primary sources of potential viral and prionic contamination come from infected animal tissue used to prepare biological reagents and media, biological products from donors (e.g. bone marrow, preembryos) and contamination during laboratory manipulation. In addition, infected laboratory workers may cause contamination of stem cell lines during culture manipulation.

Contamination by virus in "feeder" cells of animal origin

The requirement for "feeder" cells of animal origin (e.g. murine cells) to maintain undifferentiated growth in human Embryonic Stem Cells (hESCs) cultures provides intimate contact between the potential therapeutic cells and the feeder cells which are an ideal setting to transmit infectious microorganisms or bioactive molecules in the final therapeutic product. There is evidence that certain mouse viruses, like Hantaan virus, lymphocytic choriomeningitis virus (LCMV) and reovirus-3 have all been detected in mouse colonies (Kraft and Meyer, 1990) and these viruses have notably caused serious infection, and even fatalities in laboratory workers (Lloyd and Jones, 1986; Mahy et al., 1991) and may also be transmitted in cell lines and reagents (Nicklas et al., 1993). Furthermore, in the guidelines released by the European Medicines Evaluation Agency (EMEA, 1997), there is evidence that other mouse viruses like Hantaan virus, reovirus-3, Sendai virus, lactic dehydrogenase virus and LCMV are capable of infecting humans or primates. Finally, an additional group of murine viruses like ectromelia virus, Minute virus of mice, mouse adenovirus, mouse cytomegalovirus, mouse rotavirus, pneumonia virus of mice, Toolan virus and Kilham rat virus, while not known to cause human disease, are capable of replying in vitro in cells of human or primate origin (EMEA, 1997).

Contamination by virus in "feeder" cells of human origin

In the event of using feeder cells of human origin (Draper et al., 2004; Genbacev et al., 2005), there are numerous viruses and other infectious agents that are susceptible to being transmitted to the recipient because all human cells have the potential to transmit infectious diseases. The screening should apply to testing for human feeder cells for clinical use, the same as the present regulations that require the screening of cell and tissue products from donors for a spectrum of viruses which cause serious human infectious diseases (UK MSBT, 2000; AATB, 2002). Virological screening should firstly be carried out for HIV-1/2, hepatitis B and hepatitis C; moreover, recently, the products for transfusion containing cellular material have been tested for the cell associated organisms such as hCMV and HTLV-I/II (Consensus Document, 1999), although tests for hepatitis A and hepatitis E should also be recommended in such products. Other potentially viral agents that could be contaminants of cells from healthy individuals include human herpesviruses (HHV-6, HHV-7, HHV-8, Epstein–Barr virus, herpes simplex virus), parvovirus B19, TTV virus and human polyomaviruses (JC and BK virus), and these viruses remain latent and detectable in humans from early childhood. The risk of transmission of these microorganisms is considered extremely low, so tests for them are not currently required for transplantation. However, the oncogenic risk of viral contamination must also be considered because some of these agents have been involved in human cancers (Takeuchi et al., 1996; Garbuglia et al., 2003).

Some microbial agents have marked variation in their geographical distribution, producing infectious epidemics in different areas like the lymphocytic choriomeningitis virus in USA (WNV Update, 2004) and the very recent outbreak of severe acute respiratory syndrome (SARS) virus in humans in South East Asia. Furthermore, two new retroviruses (HTLV-3 and HTLV-4) have been recently identified among African bush-meat hunters (Wolfe et al., 2005) and recent cases of transplant transmitted disease due to rabies virus in USA and Germany (Srinivasan et al., 2005) have been reported, which clearly shows the potential emergence of new serious pathogens or the re-emergence of known pathogens. Obviously, any new entity that arises should be considered as a contamination risk factor, and specific tests may be required, and these may be developed for surveillance initiatives, as in the case of SARS, for which detection methods are being developed for the causative coronavirus agent (Juang et al., 2004).

Cell culture contamination by prions

The use of bovine fetal serum in stem cell cultures requires an urgent need for a risk assessment for Transmissible Spongiform Encephalopathies (TSEs) by means of a sensitive and specific test in all products derived from ruminants (U.S. Food and Drugs Administration, 1999; Directive 2004/C 24/03). Cell cultures represent a good medium to promote and to allow a persistent replication of PrP^{Sc} and to maintain the infectivity, even for heterologous cell culture models (Solassol et al., 2003). Human beings can be exposed to secondary infections of TSE using medical procedures or by administering biological products derived from humans including blood (Llewelyn et al., 2004; Peden et al., 2004). Thus, preventive measures should be taken into account with respect to blood products, cell or tissue grafts in order to avoid prion transmission in the recipients.

Methods for viruses diagnosis from cell cultures

In vitro assay for viral detection: viral culture

Viruses are obligate intracellular parasites that require living cells in order to replicate; since viruses require cellular machinery for replication, living systems must be used. The viral culture is an amplification method that increases the amount of the pathogen, facilitating detection and characterization. This method is unique among detection methods in that it provides an isolate of viable virus that can be further characterized and stored for future studies. Another important feature is that culture methods allow the detection of many different viruses, including some not suspected at the time the

culture is established; even viruses not previously known can be discovered.

A cell lysate or other specimens (e.g. biological products, specimens from donors) should be introduced into the cell culture capable of detecting wide ranges of viruses. The instructions for obtaining and transporting such specimens have been previously reported (Storch, 2001). Usually, a minimum of three cell lines that include a human diploid cell line (MRC-5), a monkey kidney cell line (Vero) and a cell type of the same species and tissue type used for production (Schiff, 2005) is recommended. Additional cells may be required depending on the cell source, passage history and raw materials used. After inoculation, cultures are incubated at 35 to 37 °C for either 14 or 28 days and inspected periodically (e.g. daily) and observed for the cytopathic effect of several viruses, including the recently reported method of analysis of HIV virus cytopathicity by using Hi-CD4 Jurkat T cells cultures (Speirs et al., 2005). In certain circumstances, the observation period can be greater than 28 days for the identification of many viruses (e.g. human cytomegalovirus).

Virus detection by testing the inoculated cell culture for hemadsorption and/or hemagglutination (Ayala et al., 2004) should be necessary for viruses with only minimal visible cytopathic effect.

However, viral culture also has significant disadvantages as a diagnostic method, including the need for specialized facilities and expertise, expense and relatively prolonged time to detection.

In vivo assay for viruses detection

The European Medicines Evaluation Agency (EMEA, 1997) and the U.S. Food and Drugs Administration (U.S. Food and Drugs Administration, 1998) recommend the use of studies in animals as a screening method for a wide range of viruses. These studies consist of inoculation in mice, pigs and embryonated chicken eggs of the cell lysate or biological products that can cause clinical changes in animals.

Species-specific viruses that are potentially present in rodent cells or stem cells with murine feeders are tested by the antibody production test like the mouse, hamster and rat test [MAP (16 murine viruses), HAP (5 viruses) and RAP (9 viruses)]. These tests are carried out by inoculating into virus-free animals and then after a specific time, examining the serum antibody levels.

However, these tests can take a number of weeks, and therefore, can prove difficult to implement in the scheduling for release testing of time critical products.

Electron microscopy

Viral infections can occasionally be diagnosed using electron microscopy for the direct visualization of viral particles in specimens. With the introduction of negative staining in the late 1950s (Brenner and Horne, 1959) and the wider availability of electron microscopes, electron microscopy became essential in characterizing many new isolates detected in cell cultures and clinical samples (Plummer et al., 1985; Biel and Gelderblom, 1999). Pattern recognition (e.g. information of size, particle morphology) leads to rapid identification of infectious agents. The “open procedure” of electron microscopic testing allows an unbiased, rapid detection of viruses and other agents if sufficiently high particle concentrations exist. Due to this capability, electron microscopic testing must be a frontline method applied to cell cultures of cultivable agents. A specimen can be ready for examination and an experienced virologist or technologist can identify, by electron microscopy, a viral pathogen morphologically within a few minutes of arrival in the electron microscopy laboratory.

Other advantages of electron microscopy include the lack of requirement for viral viability, the fact that many different kinds of viral particles can potentially be seen, and allow the differential diagnosis of many agents contained in the specimen. Disadvantages include the cost and complexity of maintaining an electron microscope (although this fact can be corrected by working with other institutions with centralized services), the need for a skilled operator and relative lack of sensitivity related to the fact that a relatively high concentration of viral particles (10^5 to 10^6 per milliliter) is required for visualization (Miller, 1995). With respect to these questions, there is a paper in which these relevant points are discussed (Biel and Madeley, 2001).

There are two types of electron microscopy methods: direct or immunoelectron microscopy. With direct methods, negative staining is normally used which requires little special equipment, in contrast to thin sectioning techniques. The specimens may be used directly or the virus particles may be concentrated before negative staining. Several methods are available for concentration, including differential centrifugation, ammonium persulfate precipitation and the agar diffusion method. Immunoelectron microscopy is a means of increasing the sensitivity and specificity of electron microscopy and is particularly useful if the number of virus particles present is small.

Details for efficient sample collection, preparation and particle enrichment have been published previously (Gelderblom and Hazelton, 2000; Hazelton and Gelderblom, 2003).

Detection of viral antigen

The detection of viral antigens directly in clinical specimens or cell cultures has become an essential component of the methodological repertoire of diagnostic virology. These methods can provide diagnostic information within a few hours of the receipt of the specimen. The lack of requirement for virus viability is another important advantage over viral culture, especially when specimen transport time is prolonged or otherwise suboptimal. Antigen detection methods can be applied when the following conditions are met: (i) viral antigen is expressed and is present in an accessible specimen, (ii) an appropriate antibody is available, (iii) antigenic variability does not preclude recognition by immunologic reagents of different strains of the target virus and (iv) the antigen being detected is sufficiently stable so that it does not degrade during transport and processing of the specimen. Methods used for viral antigen

detection include Fluorescent Antibody staining (FAs), immunoperoxidase staining (IS) and enzyme immunoassay (EIA).

Fluorescent antibody staining

FAs is widely used for detection of cell associated viral antigens. In the direct format, a fluorescent label, usually fluorescein isothiocyanate (FITC), is conjugated directly to the antibody that recognizes the viral antigen. In the indirect format, the antiviral antibody is unlabeled and is detected by a second antibody that recognizes immunoglobulins from the animal species of origin of the antiviral antibody. The second antibody carries the fluorescent label. After staining, the specimen is viewed with epiilumination using ultraviolet light of the wavelength needed to excite the fluorescent label. The direct method is simpler to use but requires conjugation of each antiviral antibody with the fluorescent label. The indirect method is slightly more sensitive and more versatile because only the antiimunoglobulin antibody has to be conjugated with the fluorescent label. The FAs method is widely used for the detection of HSV and hCMV viruses.

Immunoperoxidase staining

IS is similar in principle to FAs except that horseradish peroxidase is used in place of a fluorescent label. The advantage of this method is that the staining can be viewed by light microscopy, thus obviating the need for a fluorescent microscope. The disadvantages of the IP method are that it is more cumbersome that FAs, and endogenous peroxidases in some specimens can produce background staining.

Enzyme immunoassay

EIA is a widely used method that can be applied to the detection of antigens regardless of whether they are cell associated. Since intact cells in the specimen are not required, specimen integrity is less important than for FAs and IS. Advantages of EIA include applicability to diverse specimens and potential for automation. Viruses for which antigen EIAs have been widely used are HSV, HBV and HIV.

Serology

The measurement of antiviral antibodies was one of the first methods used for the specific diagnosis of viral infections. The role of serology in the viral diagnosis of the cell cultures may be to determine the immune status of donors of biological materials. It is important to use sensitive assays, such as EIA, immunofluorescence, Western blot assays, etc. However, research has shown that the detection of antibodies exclusively runs the risk of samples for tests being taken during an antibody-negative window period of these infections, where an individual has been exposed to viral infection and indeed can be viremic (Hitzler and Runkel, 2001). This finding has led to the introduction of nucleic acid amplification techniques, such as polymerase chain reaction (PCR), in which the presence of a virus can be observed by means of the amplification of sequences in the viral genome. The addition of nucleic acid test methods to screening of tissue donors, and to the testing of derived cell lines, should reduce the risk of these infections among recipients of stem cell lines (Zou et al., 2004).

Molecular methods

Diagnostic virology is being revolutionized by the application of nucleic acid detection techniques (Tang et al., 1997). Nucleic acid-based diagnostic tests detect only the specific virus to which the diagnostic reagent is directed. These methods detect specific nucleic acid sequences and can be applied to the detection of virtually any virus. Depending on the target sequence, the assays can be specific for a single virus species or for a group of related viruses. Nucleic acid amplification assays are particularly attractive for viruses that are difficult or impossible to culture, viruses that grow slowly in culture and viruses for which antigen detection cannot be applied because of antigenic diversity or because the level of viral antigen is too low to permit successful detection.

PCR is the prototype of target amplification assays invented by Kary Mullis in 1983 (Mullis and Faloona, 1987; Mullis, 1990). PCR is based on the ability of DNA polymerase to copy a strand of DNA by elongation of complementary strands initiated from a pair of closely spaced chemically synthesized oligonucleotide primers and includes repeated cycles of amplifying selected nucleic acid sequences (Mullis and Faloona, 1987; Mullis, 1990). After PCR amplification, the PCR product (or *amplicon*) is detected by gel electrophoresis or one of several probe-hybridization techniques, such as Southern blot.

Numerous modifications of the standard PCR procedure have been developed since its inception (Erlich et al., 1991; Wagar, 1996). Some of these modifications effectively expand the diagnostic capabilities of PCR and have increased its utility in the microbiology laboratory. RT-PCR was developed to amplify RNA targets. In this process, RNA targets are first converted to complementary DNA (cDNA) by reverse transcriptase (RT), and then amplified by PCR. RT-PCR has played an important role in diagnosing RNA containing virus infections (Young et al., 1993).

Several new PCR assays have been developed in which the synthesis of the PCR product is detected in *real-time* (*Real-time* quantitative PCR) (Gibson et al., 1996; Heid et al., 1996). Real-time quantitative PCR is a homogeneous method that includes both amplification and analysis with no need for slab gels, radioactivity or sample manipulation. Reaction products are detected with a fluorescence detection system consisting of a light-emitting diode that delivers excitation light to each reaction tube and an optical unit with three detection channels to record emitted light. The fluorescence of DNA dyes or probes is monitored each cycle during PCR. The simplest system for detection of *real-time* PCR products uses the DNA-binding dye SYBR Green, which fluoresces when its binds to double-stranded DNA. These methods have several important advantages over conventional PCR. Since the accumulation of PCR product is monitored in the reaction tube, no separate detection method, such as gel electrophoresis, is required, thus shortening the effective assay markedly. Furthermore, the possibility of contamination by *amplicons* is decreased because

the systems are closed, with no handling of the reaction contents after completion of PCR. The use of multiple fluorescent dyes with different emission wavelengths makes it possible to perform multiplex reactions with simultaneous amplification of more than one product. Moreover, other molecular methods have been described and introduced for the viral diagnosis, like nucleic acid probes (Denniston et al., 1986), Branched DNA signal amplification (Urdea et al., 1991), nested PCR (Erlich et al., 1991) and multiplex PCR (Dineva et al., 2005), etc. Real-time multiplex PCR can analyze multiple viruses simultaneously within a single reaction. The main advantages of multiplexing over single-target analysis are the ability to provide internal controls, lower reagent costs and preservation of precious samples. Multiplexing can be particularly important when there is a need to analyze several viruses from the samples. There are several assays that use a real-time multiplex RT-PCR technology for diagnosis of hepatitis B virus, hepatitis C virus and HIV-1 virus (Candotti et al., 2004). There are other authors who have described several assays using this procedures for retroviruses (Vet et al., 1999) and herpesviruses (O'Neill et al., 2003).

The advantages of molecular methods, especially the PCR technique, are their extremely high sensitivity (they may detect down to one viral genome per sample volume), they are easy to set up and have a fast turnaround time.

However, the main inconvenience is that for each virus or group of virus one PCR is necessary, so if the amount of viruses to carry out is large, these techniques are the same unviable for the laboratory.

Test for retroviruses

Retroviruses are one of the main contaminants of the cell cultures. For these viruses, reverse transcriptase assays, electron microscopy techniques and infectivity assays must be included. A variety of infectivity assays are available for rodent cell lines or stem cell lines with murine feeders. There are two retrovirus infectivity assays for the ecotropic and xenotropic viruses: XC plaque assay using indicator cells (XC) to form syncytia (plaques) for detection of ecotropic viruses (Lenz and Haseltine, 1983) and mink S^+L^- assay for the detection of xenotropic viruses (Li et al., 1999). However, these tests are not suitable to detect and quantify the levels of the ecotropic recombinant virus, thus a serological focus assay, based on specific antimurine leukemia virus (MuLV) viral envelope antibodies is required to detect ecotropic recombinant virus (Deo et al., 1994). Moreover, for low levels of murine retroviruses, amplification may be achieved using cocultivation of cells with a susceptible cell line such as *Mus dunni* cells.

The reverse transcriptase assay is an enzymatic technique to detect the presence of extracellular retrovirus particles. This assay is based on the ability of reverse transcriptase associated with retroviruses to synthesize radiolabeled nucleotides into complementary DNA (cDNA) copied from synthetic templates. Due to the fact that a variety of enzymes are capable of incorporating labeled deoxynucleotide into an acid-insoluble material, this assay is susceptible to false positives. A useful test for eliminating false positives is to compare the results from both a DNA template and a ribonucleic acid (RNA) template because cellular DNA polymerase and viral reverse transcriptase have different template preferences. Furthermore, the PCR-based reverse transcriptase assay is more sensitive than the standard enzymatic reverse transcriptase assay.

Electron microscopy (see below) is used to visualize both the ultrastructural morphology of the cell substrate and the presence of virus and virus-like particles.

Microarrays methods for viruses

The development of microarrays has been fueled by the application of robotic technology to routine molecular biology, rather than by any fundamental breakthrough. Southern and Northern blotting techniques for the detection of specific DNA and mRNA species provided the technological basis for microarray hybridization.

The construction of arrays involves the spotting of specific DNA sequences on a glass slide or small silicon chip by photolithographic combinatorial chemistry methods similar to those used to make electronic chips. Membrane-based arrays may be in the format of line probe blots. The results of hybridization between the bound probe and labeled sequences in the sample applied and tested are revealed by scanning or imaging the array surface. Confocal microscopy is used to scan the chip, detecting fluorescent signals that reveal hybridization at precise locations on the chip. Use of probes representing all possible nucleotide sequence variations within a target sequence allows rapid determination of nucleotide sequence (Pease et al., 1994). High density arrays, which may have thousands of individual probes per cm^2, are referred to as microarrays. These microarrays on silicon surfaces are therefore known as "DNA chips". As many DNA sequences can be present on a slide, it is possible for microarray analysis to test for multiple pathogens (including viruses) simultaneously.

The first application in diagnostic virology has been for rapid sequencing to detect HIV mutations associated with resistance to antiretroviral drugs (Kozal et al., 1996). Other roles in virology are in diagnosis, to recognize the causative agent of an illness; for molecular typing (e.g. patient management, epidemiological reasons, purposes related to vaccine use) and in research, to investigate the interactions between the virus and the host cells (Clewley, 2004). The RNA expression of human CMV in cell culture in the presence or absence of cycloheximide or ganciclovir was analyzed with an array of oligonucleotides representing human CMV ORFs (Chambers et al., 1999). Other groups have developed microarrays that detect simultaneously and discriminate several viruses like orthopoxvirus species (Laassri et al., 2003), respiratory viruses (Coiras et al., 2005), herpesviruses, enteroviruses and flaviviruses (Korimbocus et al., 2005) and hepatitis C virus (Xu et al., 2005). Also, Wilson et al. (2002) developed a multi-pathogen identification microarray system for the identification of 18 pathogenic prokaryotes, eukaryotes and viruses.

The simplicity of the microarray protocols, together with their use of a large number of species-specific oligoprobes and their ability to analyze multiple samples in a short time, offers clear advantages. However, such techniques will need to be carefully validated for sensitivity and specificity before being applied in the safety testing of human tissue products and stem cell lines for therapy.

Table 1 shows a comparison of diagnostic methods for viruses.

Proteomic analysis for viruses

Proteomics also holds a key position in the new functional genomics biology and is a term for large scale analysis of proteins. Proteomics encompasses different methods to identify all the proteins present in a cell or tissue.

Protein arrays are being prepared with antigens or antibodies bound to a solid phase (analogous to oligonucleotide or amplicon probes) and used to capture specific antibodies or antigens (Emili and Cagney, 2000; Walter et al., 2000). Essentially, these are immunoassays in a microarray format (Schweitzer and Kingsmore, 2002). A protein array has been described for ToRCH screening and *Toxoplasma gondii*, rubella virus, CMV and HSV-1 and 2 antigens were printed on glass slides. The slides were first incubated with serum samples and subsequently with fluorescently labeled secondary antibodies. Human IgG and IgM bound to the printed antigens were detected by confocal scanning microscopy. Good concordance was obtained between the microarray results and those of ELISAs (Mezzasoma et al., 2002). A microarray of oligosaccharides on nitrocellulose has been developed to capture carbohydrate-recognizing proteins, and it might be possible to develop this for viral diagnosis (Fukui et al., 2002).

Biosensors have been defined as small devices which use biological reactions to detect target analytes (Wang, 2000). There are two ways of doing this; bioaffinity arrays involve the target analyte binding to a ligand immobilized on a solid phase (e.g. an oligonucleotide) and, instead of there being a necessity for detection of hybridization of the probe and target by colorimetric or radioactive means, the hybridization is detected by electronic means. Biocatalytic arrays involve an immobilized enzyme being used to recognize the substrate of the enzyme, which is, in this case, the target of the array. A signal is generated when the enzyme catalyzes a specific reaction because of the presence of the target in the analyte applied to the array. The reaction may be recognized either by colorimetric ally or via an electronic transducer at the surface of the array.

With more technological advances, protein/antibody arrays are likely to impact initially on infectious disease research with profiling sera, body fluids to discover diagnostic markers of particular infections. Proteomic techniques also will offer the potential for discovering markers for diagnostic tests of viral infections in vitro (e.g. stem cell cultures).

Diagnostic methods for prion particles

An interesting potential for infected cell cultures may be the discovery of biological markers of prion infection, mainly by comparing control versus infected cultures. This type of experiment was first performed at a genetic level. With the development of sophisticated proteomic approaches, several groups are looking for differentially expressed proteins that could be used as diagnostic markers or at least could give some clue as to the physiopathological event leading to prion propagation. Having developed cell lines highly susceptible to prion infection (Bosque and Prusiner, 2000; Nishida et al., 2000), another potential of cell culture consists of the detection of infectivity in various biological samples.

The main sources of transmission of prion proteins to culture cells are the bovine fetal serum, the feeder cells from murine animals and the biological products from donors with TSE to establish stem cell lines. In stem cell banks, there is an urgent need for tests for the agents of TSEs such as the Creutzfeldt–Jakob disease (U.S. Food and Drugs Administration, 1999). At the moment, there are several types of tests that can be used for this question.

Detection of antibodies from prion proteins

The cell prion protein (PrP^c) is essential for pathogenesis and transmission of prion diseases (Prusiner, 1989). During the

Table 1
Comparison of diagnostic methods for viruses

Method	Advantages	Disadvantages
Experimental animals	Only method available for some viruses; used to study pathogenesis	Expensive; slow; complex systems–complex results
Cell culture	Used to study and quantify most viruses; sensitive	Slow; technically demanding
Antigen detection	Rapid; for viruses which cannot be cultured	Technically demanding; equipment is expensive
Serology	Rapid; sensitive; can be quantitative; automation	Difficult interpretation; sensitivity variable
Nucleic acid-based	Rapid; sensitivity	Expensive
Microarrays	Used for a big quantity of viruses	Sensitivity and specificity non demonstrated; absence of evaluation
Electron microscopy	Rapid; sensitivity; teaching value; absence of viral viability; identification of new agents	Cost elevate; complexity of maintaining; requires trained operator; requires high concentration of viral particles

course of prion disease, a largely protease resistant aggregated form of PrP, designated PrP^{Sc}, accumulates mainly in the brain, and may be the main or only constituent of the prion (McKinley et al., 1991). The detection of this protein can be carried out by means of antibodies of the monoclonal IgG1 subtype, anti-PrP 6H4 (Enari et al., 2001; Parizek et al., 2001); this antibody recognizes the sequence DYEDRYYRE in the prion protein (human PrP: amino acids 144–152). This sequence is conserved in most known mammalian PrP sequences (human, cattle, sheep, rabbit, mink and a variety of primates). 6H4 can be used for Western blotting and ELISA (Prionics, 2002). There are other monoclonal antibodies for prion protein such as 34C9 that recognizes the sequence LIHFG in the bovine prion protein and a polyclonal antibody R029 that also recognizes bovine PrP.

Recently, Sanquin reagents (Sanquin, 2005) has developed a new monoclonal antiprion antibody for use in research applications (clone 1E4). In contrast to many other antibodies used for detection of prion protein, 1E4 has a broad species reactivity. Detection of prion protein has been demonstrated with Western blot for humans, cattle, sheep, deer, mice and hamsters. Most of the currently available TSE tests are based on the fact that PrP^{c}, normal prion protein, is digested by proteinase k, whereas PrP^{Sc}, TSE specific prion, is relatively resistant to degradation by proteases. The special feature of 1E4 is that its epitope is almost hidden on non-digested bovine PrP^{Sc}, but after proteinase K digestion, the epitope becomes available resulting in a significant increased detection. After digestion with protease K, 1E4 binds to PrP^{Sc} with high affinity, whereas it has a low affinity for non-digested PrP^{Sc}. The 1E4 antibody has been tested in a broad variety of methods, such as Western blot, RIA, ELISA, EliBlot, FACS and immunohistochemistry.

Cyclic amplification of protein misfolding

Saborio et al. (2001) have developed a procedure, conceptually similar to polymerase chain reaction cycling, involving cyclic amplification of protein misfolding PrP^{Sc}. This method could allow a rapid conversion of excess PrP^{c} into a protease resistant PrP^{Sc}-like form. In this method, aggregates formed when PrP^{Sc} is incubated with PrP^{c} are disrupted by sonication to generate multiple smaller units for the continued formation of new PrP^{Sc}. After cycling amplification, more than 97% of the protease resistant PrP present in the sample correspond to newly converted protein. This method could be applied to diagnose the presence of undetectable prion infectious agent in tissues and biological fluids. In this respect, recently, Castilla et al. (2005) have been the first to detect the resistant-protein PrP^{Sc} biochemically in hamster blood by means of the cyclic amplification of protein misfolding. This procedure enables detection of prions in blood with 89% sensitivity and 100% specificity. The high level of sensitivity and specificity indicated that this assay offers promise for the design of a sensitive biochemical test for blood diagnosis of transmissible spongiform encephalopathies. The implementation of a similar blood-detection procedure for humans and culture-detection method for stem cell lines undoubtedly contributes to minimizing the risk of infection with agents causing transmissible spongiform encephalopathies.

In vivo assays with transgenic mice

The diagnosis of infections produced by TSEs can be carried out by means of the detection of prionic protein (PrP^{Sc}) by different methods (see below). Although the specificity of these diagnostic methods is nearly 100% (Ironside, 1996; Lee et al., 2000; Wadsworth et al., 2001), the sensitivity is still inadequate to assure the value of a negative result. An alternative to these methods would consist of the use of transgenic mice; these would be vaccinated with tissues or fluids that have unknown infectivity of the cell lines which have used bovine serum to be obtained.

Conclusions

Stem cell banks arise from the necessity to guarantee the existence of an appropriate source of cell lines in a standardized way for their use in research or human therapies through clinical trials. Moreover, these establishments should assure the safety of biological products for use in cell therapy.

The major risks associated with the use of biological products in regenerative medicine are related to cell contamination. These include both serious human pathogens but also human and animal viruses and prions capable of multiplying and producing transmissible infectious diseases. Selecting and testing of stem cell lines and biotechnological products (e.g. bovine serum, tripsine, culture media) is one part of a strategy for establishing a viral safety program (Cobo et al., 2006). While the technology to avoid the animal products in the

Table 2
Examples of specific viruses that may be transmitted in transplanted cells and cause serious diseases and currently available screening tests

Viruses	Diagnostic tests
Viruses of human origin	
HIV-1/2	NAT, serology[a]
HBV	NAT, serology[a]
HCV	NAT, serology[a]
HTLV-I/II	NAT, serology[a]
CMV	NAT, culture, EM, serology[a]
EBV	NAT, serology[a]
Prions	WB, ELISA
Viruses of animal origin	
BVDV	NAT (RT-PCR), EM
Hantaan virus	APT, EM
Reovirus-3	APT, EM
Sendai virus	APT, EM
Lactic dehydrogenase virus	APT, EM
Lymphocitic choriomeningitis virus	APT, EM
Prions	WB, ELISA
Unknown viruses	EM

NAT: nucleic acid test (PCR, RT-PCR); EM: electron microscopy; APT: antibodies production tests (MAP, RAP, HAP); WB: Western blot; ELISA: enzyme-linked immunosorbent assay; BVDV: bovine viral diarrhea virus.
[a] Serology: for donors of biological products.

Table 3
Virus tests recommended in the characterization of cell lines (modified from Dellepiane et al., 2000 and EMEA, 1997)

Test	Master bank	Work bank	Cell at the limit
Electron microscopy	+	–	+
Reverse transcriptase[a]	+	–	+
In vitro cell inoculation	+	–	+
In vivo animal inoculation	+	–	+
Antibody production tests[b]	+	–	–
Infectivity[c]	+	–	+
Other virus specific tests[d]	+	–	+

[a] Not necessary if positive by retrovirus infectivity test.
[b] e.g., MAP, RAP, HAP—Usually applicable for rodent cell lines.
[c] For retroviruses and other endogenous viruses.
[d] Tests for cell lines derived from human, non-human primate or other cell lines as appropriate.

culture manufacturing is being developed, the safety of the human and/or animal products used in the cell cultures with respect to viral and prion contamination could be obtained for application of an exhaustive program of viral screening by means the combination of techniques above-mentioned (Tables 2 and 3). The presence of viruses (including non-pathogenic types) or prions in a human therapeutic product would most likely render it unacceptable for clinical use.

At the moment, the cell banks must have a panel of tests to detect serious pathogens like endogenous viruses, exogenous viruses and prions. This panel of tests should necessarily include reverse transcriptase detection as a general test for retroviruses, electron microscopy that can detect different kinds of viral particles and characterize many unknown isolates present in cell cultures and molecular techniques like PCR (conventional or real-time) and RT-PCR tests to include all the viruses that we know pose a risk to the product. For prion detection, these banks must have a procedure based on the Western blot or ELISA technology for the detection of antibodies (e.g. 6H4, 1E4) that can be present in the cell cultures. Some of these tests may also need to be applied to culture reagents of animal origin, and any testing performed by the manufacturer should be carefully evaluated before accepting the reagents for use.

With respect to the use of "feeder cells" of animal origin (e.g. murine) for embryonic stem cell culture, there is a need to evaluate several viruses that are capable of infecting humans or primates and that potentially can cause serious infections in laboratory workers. For these viruses, the antibodies production tests (e.g. MAP, RAP, HAP) are the most convenient procedures in addition to the evaluation by means of electron microscopy.

Several tests could be included in the screening of other biological products for a wide range of viruses: the in vitro cell culture assay and the study in animals (EMEA, 1997). These are long standing techniques that rely on the ability of many viruses to cause cytopathic changes in cell culture or clinical changes in animals. However, there is a difficulty for implementation in most of the laboratories due to the need for specialized facilities and expertise, relatively prolonged time to detection (e.g. can prove difficult to implement the testing of time critical products) and the high cost.

In the immediate future, the technologies based on the use of hybridization chips, using microarrays of immobilized oligonucleotides or antigens/antibodies for viruses, and the method of cyclic amplification of protein misfolding PrP^{Sc} for prions, can provide a rapid and useful methodology of the identification of contaminants. In our opinion, there is a need to design and to implement this methodology in stem cell cultures, so new research will be needed.

Finally, it is not possible to assert the absolute absence of viral contaminants because of both the impossibility of covering all potential viral contaminants and the absence of adequate levels of sensitivity and specificity of diagnostic techniques in several cases. So, stem cell banks could potentially permit transmission of some of these viral contaminants to many patients receiving future stem cell therapies where the balance of risk and benefit may be different.

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