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Biosafety of Mammalian Cell Cultures

Proceedings
Basel Forum on Biosafety
28 October 1993

Agency ***BATS***
Clarastrasse 13
CH-4058 Basel

Phone ++ 41 61 690 93 10

Fax ++ 41 61 690 9315

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BATS, Agency for Biosafety Research and Assessment of Technology Impacts,
Clarastrasse 13, CH-4058 Basel / Switzerland
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W. Berthold
Dr. Karl Thomae GmbH, Biberach/ Riss, F R G.

Applications of mammalian cell cultures : Setting the scene

Introduction

The progress in the civilisation of man has been marked by inventions of new technolgies that facilitate the well being of man kind always in close neighbourhood to the creating of new potential risks. One of the oldest inventions is the use of microorganisms for the preparation of food like bread, milk products, wine and beer and other forms of preserves. Although the knowledge of the mechanisms involved have only been detected in historcally recent years, the technology has been perfected empirically quite well. The resulting products have been well accepted despite the ignorance of what and how microorganisms are involved in the process. This is amply demonstrated by the preference of many people for a mode of beer making practised and legally regulated in bavaria since the 16th century according to the imperial verdict of the "Reinheitsgebot" (purity law). At that time the concept of such small "animals" or of chemical elements had clearly not been developed.
Only in the last century it became apparent, how plentifull the microscopical world of microorganisms is, and that certain strains are related to (infectious) diseases (Discoveries of Koch for tuberculosis and Pasteur for anthrax etc.). For the first time in history, the possibility was created to search for the propagation of individual strains/cells rather than dealing with mixed populations.
It had also been discovered in the 17th century with the invention of the microscope, that animals consist of many individual small "cells", the latin word for a confined small room.
These discoveries were the foundation on which the isolation and in vitro propagation of animal cells could be addressed.

Why should mammlian cells be kept in vitro ?

The early days

But what has been the motivation to try to take cells normally growing in tissues and whole organisms out of their natural habitat? In the early days, the strongest motivation certainly was scientific curiosity namely the desire to understand better what the functions of cells are and how they contribute to the functioning of the entire organism. Thereby, the difference between single cell organisms and animals should become apparent. Even today, this remains a dominant driving force for cell cultivation.
In the first half of this century a famous experiment was the maintenance of chick embryo cells in culture for a long time (Cajal). The cell appeared to enjoy some immortality or rejuvenating potential. This phenomenon was later questioned and today, it seems likely that the culture medium, prepared from mashed chick embryos in which all chick cells were thought to be dead or removed, may still have contained young primary cells. This episode stirred up much scientific

discussion on longevity of cells in extracorporal growth conditions compared to their natural surroundings in the animal.

Vaccine production

By progress in the discovery of microorganisms it was learned how to differenciate between infectious single cell organisms capable of self-replication and even smaller entities, that require a cell for amplification, but could not self-replicate and could pass filters that would retain bacteria: phages and viruses.

The success of immunisation of animals with inactivated and no longer infectious pathogens that Pasteur had demonstrated spurred the effort to use the same procedure for viruses. Not knowing the reason, Jenner had already made use of the observation, that a protection against small pox could be obtained by the treatment of people at risk with lymph (or 'virus', i.e. a disease transmitting fluid) from infected cows in 1798. The word vaccination is now used synonymously with active immunisation using an attentuated or related, but not pathogenic pox virus strain. This description of the procedure and the rationale behind it required some 150 to 200 years of biological and medical research.

The propagation of virus strains suitable for vaccination was first found possible in animals. However, it was desirable to develop cleaner systems, where the pathogen could be propagated under better control, and be prepared without the other antigens originating from the animal donor, but not necessary to transfer immunity.

The first virus vaccine preparations obtained by mammalian cell cultures were not very pure virus. Cells were cultivated in a nutrient medium that did have to contain rich and crude biological extracts such as foetal bovine serum as essential element for cell maintenance and growth in culture. The resulting virus harvest consisted of the crude cell supernatant. A purification of virus particle was usually not possible. The desired 'active' ingredient in the vaccine constituted virus particles by far outnumbered in amount by cellular proteins (and DNA) as well as bovine serum components

Cultured mammalian cells today's perspective

Vaccine production was the first important product derived from cell cultures. The cells were grown in the adherent mode, which faciltates the recovery of cell-free supernatant. Other natural proteins such as fibroblast interferons could be produced and have reached the market place.

Antibodies become a dominating topic after the discovery of immortalization of B lymphocytes by cell fusion with a naturally derived mouse myeloma cell P3. These cells were grown in suspension culture. The design of large scale equipment for fermentation of microorganisms had been well developed then. Threfore, a rapid orientation occurred for large scale production towards suspension culture even of cells that normally grow adherently such as CHO cells.

The biotechnology of animal cells obtained a new perspective, when recombinant DNA techniques could be applied also to mammlian cells. The full potential of this field has not been explored yet. It can be expected, that there will be production of rare and valuable proteins by recombinant cells (cytokines, enzymes, hybrid proteins etc.), multi protein systems, virus-like particles and even whole, special cells like in gene therapy of cancer patients and tissue-like two or three-dimensional 'organs' will be generated in vitro. This development will expand and diversify the use of mammalian cell culture enormously. The technical challenges

will be on two levels, the optimization of the cell's biological properties (special proteins, metabolic pathways, nutrient and substrate requirements) and the technical improvement of the cell culture instrumentation and the production line equipment adapted to the scale and special requirements.

Topics related to risks :

Who may be at risk ?

Risks arising from mammalian cells in culture can generally be classified into three categories:

- risks for operators during cell cultivation
- risks for the recipient of the product of the cells
- risks for the environment in general, including animals and plants.

It is very difficult to define a risk for the environment that originates from cells in culture that not already occurs in the 'wild' or the animal species from which the cells are derived.

The potential biological risks for operators and recipients may well be of similar nature, but different in variety and intensity. While the operator may be in superficial contact with cruder, less well defined biologicals during cell cultivation, the final purified product is usually applied to the recipient in a parenteral mode, penetrating artificially the natural barrier of immunity.

Nature of potential biological risks of cells in culture

Virus production was the first example for material derived from cells in culture to be given to humans as medication. The safety for the patient is of great importance. At that time, the discussion concentrated on the properties of the production cell. It was considered safe to use diploid primary cells from healthy donors (human and animal) for virus replication most notably to produce inactivated polio vaccine. Examples are primary kidney cells from rabbit, hamster and green monkey and only later of human (foetal) fibroblasts. A well known human diploid cell line is WI-38

The reason for the choice of animal cells was the idea, that human cells may contain human oncogenic agents, while the equivalent animal material was thought not to influence humans. In addition residual DNA caused concern, because it was assumed that the endogenous oncogenes could be transmitted to the patient. These facts influenced testing of cell lines in two ways:

1. The monitoring of oncogenes in residual cellular DNA was abandoned, when it was discovered that protooncogenes are important normal genes found in every cell.
2. Potential hazard from DNA lead to the regulatory requirement to lower the level of DNA content to below 100 pg DNA per dose of protein product. A problem remained for vaccines especially DNA virus vaccines, where the active ingredient can not easily be distiguished from the cellular DNA.

However, during the last years, concern has not been substantiated by any abnormally high incidence of tumor formation. Infact, the testing for tumorigenicity of continuous production cell lines in immune suppressed animals has largely been dropped as a regulatory requirement, because all such cell lines can grow as tumors in immune compromised animals and this demonstration provides no relevant safety aspect.

Later, endogeneous virus became a dominant concern. It also became apparent that many primary cells have a limited life only in vitro and could not be tested very exhaustively for latent virus infections during the available in vitro life span.
However, in order to have enough cells that can be tested sufficiently, a strategy of cell banking for long living cell lines was promoted. This meant, that suitable cell lines had to be developed. A cell line is defined by the properity of the population of cells to maintain cell divisions for over 70 passages. Such cell lines became acceptable as substrate for virus production, after detailed discussions on risks related to in vitro immortality being characteristic for cancer in vivo.

Concept of testing and validation
Concerns with regard to virus relate to two categories of viruses a) (latent) infectious viruses, and b) tumor viruses. The presence of retroviruses and retrovirus like particles in continuous cell lines is now well known. Although, it is obviously desirable to use cell lines where no retrovirus is found, many lines especially hybridoma cell lines have been found to contain such structures. Today it appears, that the retroviral sequences are very abundant even in animals. So that it may be 'unnaturally' not to contain retrovirus related genetic elements in any natural cell. Rather than searching for alternative but unlikely type of risk-free cells, a strategy of product purification especially designed to remove and/or inactivate retro- and other viruses has been adopted for protein products. The efficacy of such procedures is demonstrated by validation using a number of different model viruses in spike experiments. Monoclonal antibodies have now become accepable safe products, despite the presence of retroviral particles in the master cell bank cells.
The situation today is that continuous cell lines are well accepted and can be used for the generation of medicinal products, if absence of infectious agents such as a long list of viruses, mycolplasma and other microorganisms (= sterility) is proven (in the master cell bank), adequate virus removal/inactivation can be demonstrated in the purification process by validation experiments and if adventitious infectivity assays are negative.

"Historical" evaluation of risks of mammalian cell cultures

I have chosen a number of examples for historical situations, when scientific rationale (microorganism processed food) or technical performance (early vaccine preparations) were still adequate for the acceptance of the product. I am not aware of studies or incidences that have correlated any serious disaster with handling modes of the corresponding time period. Of course, it could be argued that such incidences could not be detected because of the scientific views or ignorances of the days. However, what ever did happen it was accepted as unavoidable fate, rather than a lack of diligence.

I would like to take these examples to place todays wisdom, especially todays perceived risks into a larger frame of reference. Although, we know so much more and therefore can consider so many more possible risks, we should not ignore the fact, that the likelyhood of the vast majority of such risks is very small indeed.

We should not overemphasize postulated, more hypothetical risks and play down real risks only because we have been accustomed to the situation or because we can not avoid them in our regular live or activities (carcinogenic benzine in car petrol, road accidents, alcohol and tobacco related hazards, infections when travelling in the tropics etc.).

It appears to me that viral infection remains the most serious potential risk associated with mammalian cells in culture. This type of risk is not new, and the potential pathogens are 'natural' and well adapted by natural selection and highly evolved biological entities, that are more sophisticated than our man made recombinant DNA. An example for our limited understanding is the unravelling of the HIV functions. It should be noted that never before was the time so short between discovery and the illucidation of genetic content of a human pathogen than for HIV. And still, this basic knowledge has not been sufficient for the expected success in terms of a possible cure.

Despite the simple technology initially used for putting mammalian cells into culture, requiring e.g. many ill characterised biologicals, there is no indication for a serious biohazard associated with the technology. Todays procedures and efforts for standardisation will lead to even more predictable and therefore safer procedures in cell culivation. The vast increase in possible applications of mammalian cells promoted by recombinant DNA techniques do not change the intrinsic biological risk of cells in culture to beyond those unexcludable risks that have and will always be associated with life.

Benefits derived from cultured cells

Above all, cell culture will continue to provide the tools for discovery in bio-medical research. Screening and toxicological assays on cells in culture will permit a better understanding of normal and diseased functions of the body and thereby help to find better drugs and test safer remedies involving less animals in this research. This may be expected for the complexity of the immune system and of neurobiology using highly sophisicated in vitro cultivation techniques.

At large scale cell cultures have been developed to produce more specific, less risky vaccines, live saving (e.g. tPA) and live sustaining medicinal proteins (e.g. insulin, EPO). In the future they may even be used for regeneration of skin or extracorporal artificial organs.

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Volker Kamla, Birgit Henrich
Institute for Medical Microbiology and Virology, Heinrich-Heine-University, Düsseldorf, F.R.G.

Hazard Potential from Mycoplasmas

Mollicutes, often named mycoplasmas, are a group of extremely small bacteria. Some species are plant (Seemüller 1989) and insect (Clark 1984) pathogens, others cause veterinary and human diseases, and many colonize plants, animals or man as apathogenic commensals. (Nocard 1898, Müller 1983, Lo 1989). Cultivating of most mycoplasmas in artificial media requires several supplements i.e. cholesterol. With the development of eukaryotic cell-cultures containing these supplements, mycoplasmas have become recognized as potential culture-contaminants (Del Giudice 1978, McGarrity 1985a). With the recent expansion of cell-culture in biotechnology the contamination by mycoplasmas has become a new meaning. As easy as contamination occurs, is its detection and elimination difficult. In contrast to direct visible contaminations with bacteria or fungi which overgrow and kill the cells within days, mycoplasmas are able to persist in permanent cell-cultures undetected for years.

How do they enter cell cultures

As a source of mycoplasma contamination several factors are discussed:
- spreading by handling of a previously contaminated cell-line (the so-called "kind gifts" from other laboratories are in fact often "trojan horses"),
- contamination with human commensal mycoplasmas from laboratory personnel,
- import to the laboratory by establishing a primary cell culture of an infected animal,
- use of contaminated supplements (serum, cytokines, supernatants of infected cells),
- splitting adherent cells with contaminated trypsine.

In cell cultures mycoplasmas reach concentrations up to 10^7-10^8 cfu per ml and a contamination, once established in the primary cell culture, spreads easily to all others by droplet infection.

Why should we care about them

Mycoplasma-infected host cells can change several properties: growth, metabolism, morphology and genome structure (McGarrity 1985b). The bacteria also compete with host cells for nutritients. Mycoplasmal acidic or alkaline metabolites decrease the growth of host cells. Degradation of arginine and purines by mycoplasmas may inhibit the synthesis of histone and nucleic acid, followed by chromosome aberrations of the host cells. Some mycoplasmas produce hydrogen peroxide, which is directly toxic to cells.

Mycoplasma effects are never predictable, thus effects caused by a mycoplasma-strain in one given cell line may not occur in another cell line infected with the identical strain. The infected cells normally show decreased growth and cytopathic effects (McGarrity 1984), but some cell lines grow faster. Virus propagation in mycoplasma infected cell cultures can lead to a decreased or increased yield of viruses (Hargraves 1979). Mycoplasmas can, but do not necessarily, reduce tumorgenicity of tumor cells in vivo. They are able to alter cytokine expression of some host cells. Mycoplasmal enzymes reduce [^{3}H]-thymidine incorporation in bioassays (Sinigaglia 1985). Oxygen-consumption continues in infected cell lines after treatment with cyanide (Koch 1983). Therefore mycoplasmal contamination may interfere with all test systems based on cell cultures.
All the above facts clearly demonstrate that the correct interpretation of results requires regular contamination control.

How can we detect a mycoplasma contamination

The methods of reliable detection of contaminating mycoplasmas are fastidious. The cultural detection is very sensitive but it requires up to 2 weeks and several strains (e.g. M. hyopneumoniae and some strains of M. hyorhinis) can not be cultivated. Direct DNA-staining with intercalating fluorescent substances (Russell 1975, Chen 1977), e.g. DAPI or Hoechst 33258 is a fast method, but it may fail on non-adherent mycoplasma strains.
Another method is the selective killing of mycoplasma-infected cells in presence of 6-methyl purine deoxyriboside (6MPDR) (McGarrity 1982), but not all cells are susceptible. Several authors describe the direct vizualization of mycoplasmas by electron microscopy (Takayama 1983, Barth 1988) or enzyme-immunoassay with specific antisera or monoclonal antibodies (Douma 1989, Blazek 1990).
Detection by ^{3}H-labelled DNA to mycoplasmal rRNA (Goebel 1984) is very sensitive but fails on some mycoplasmas. A more recent method is the PCR-amplification of mycoplasmal rRNA (Spaepen 1992).
Since there is no single method for all cells and all mycoplasma strains, parallel testing with two or three different methods are strongly recommended to safely exclude mycoplasma contamination.

How can we eliminate mycoplasmas

A palette of methods for the elimination of mycoplasmas from cell cultures is described, including selective damage of mycoplasmal DNA with 5-bromouracil or DNA-staining substances (Hellkuhl 1983); in vivo passaging of tumor cells in nude mice; cocultivation with macrophages or antibiotic treatment. The increasing number of fluorinated quinolone-derivatives, which are mycoplasmocidal (ciprofloxacin, mycoplasma removal agent) demonstrates that antibiotic therapy gives acceptable results. Although fluorinated quinolone-resistant mycoplasma strains have been reported, most failures of treatment are a result of an immediate re-infection.
In summary no optimal elimination method exists, as most methods can either affect host-cell properties or they are time- and work-intensive or are poorly effective.

The best and the oldest way to eliminate mycoplasmas remains the discarding of infected cell-lines.

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H. Diggelmann
Institute of Microbiology, University of Lausanne, Lausanne, Schweiz

Hazard potential from viruses and oncogenes

To evalutate the risk potential of an infectious agent it is necessary to know its characteristics, its natural hosts, its modes of transmission and its disease potential. According to these different criteria, viruses, like other infectious agents, have been classified into four different risk groups and the corresponding working conditions have been defined [1]. Based on these informations it is relatively easy to be aware of the risks associated to experiments with a given virus in a well defined in vitro culture system. In cases where an effective vaccine is available vaccination of the laboratory personnel is recommended.

Genetic modifications of viruses might result in changes in their replication potential, their host range, their immunogenicity and their pathogenicity. Such changes have to be taken into account in the risk assessment of an experiment.

The use of primary cultures from animal or human sources adds an additional element of risk to all experiments. Such cultures can be contaminated with a large number of infectious agents. The presence and release of such infectious material can not only be hazardous by itself, but in certain cases recombination events or phaenotypic mixing might occur between contaminating components and the experimentally introduced organism, creating infectious agents with new properties. These possibilities have to be taken into account in the risk assessment.

The exposure of living organisms to naked nucleic acids is generally considered harmless. The possibility to produce large amounts of cloned DNA molecules harboring strong transcriptional promoters and genes coding for potentially harmful functions might require a slightly more conservative attitude. It has been recently shown that intramuscular injection of very large amounts of such expression vectors leads to expression of the genes and an immune response [2]. The pathogenic potential of gene products coded by such expression vectors should therefore be taken into account in the risk assessment.

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Summary of the transparencies

Main viral contaminants in human tissues:

- Hepatitis Viruses:
  HBV, HCV, HDV, HEV
- Human Retroviruses
  HIV-1, HIV-2, HTLV-1, HTLV-2
- Herpesviruses:
  EBV, CMV, HHV-6, HSV-1 and 2
- Popovaviruses:
  different HPV sequences, but no virus production
- Prions:
  Infectious prion proteins

Viral contaminants in primate tissues causing human disease

RNA Viruses:

Flaviviruses (Yellow Fever virus, Kyasanur Forest virus)

Filoviruses (Marburg and Ebola virus)

Simian hemorrhagic virus

Rabies virus

Hepatitis A virus

Poliovirus

DNA-Viruses:

Herpesviruses (Herpes-B-Virus and others)

Molluscum Contagiosum

SV40 (non pathogenic for humans)

Only a few viruses of mice are pathogenic for humans

Lymphocytic choriomeningitis virus (LCMV)

Hantaan virus (hemorrhagic fever with renal syndrom)

Sendai virus (murine parainfluenza virus type 1)

A significant percentage of the mammalian genome is made up of endogenous retroviral sequences

- endogenous retroviral sequences are part of the normal genetic makeup of an organism (1-5% of total genomic DNA)
- some of these sequences represent complete retroviral genomes, but a large number of proviruses are defective
- the majority of endogenous retroviral sequences are not transcribed
- some of the endogenous retroviral squences are transcribed but do not code for functional proteins
- some endogenous proviruses code for functional proteins that might play an important role for the host (e.g. MMTV superantigen)
- endogenous retroviral sequences can recombine with exogenous retroviruses or complement defective exogenous retroviruses
- it is thought that endogenous retroviral sequences play a role in evolution
- endogenous retroviral particles can be released from cultured cells, their host range can vary and some of them infect human cells in culture. No endogenous retroviruses pathogenic for humans are known.

Exogenous Retroviruses

- exogenous retroviruses can be replication competent or defective
- defective viruses have survived in nature as their defects can be complemented in presence of a replication competent exogenous virus serving as helper virus or by gene products of endogenous retroviruses. The result of such infections are phenotypically mixed virus particles
- some exogenous retroviruses carry transforming genes (oncogenes) and cause tumors rapidly and at high frequency. No such human retroviruses have been identified
- The random integration of exogenous retroviruses into the host chromosomes leads to insertional mutagenesis. With a low frequency such an integration event can activate a cellular oncogene and lead to tumor formation
- some exogenous retroviruses code for transcription factors that affect the expression of cellular genes e.g. involved in the control of cell growth
- because of their strong species specificity only human retroviruses are considered pathogenic for humans

W. Slenczka
Philipps-Universität, Marburg, FRG

Case Study: Occupational infection with Marburg virus.

Marburg virus (MBG virus) was detected in late summer 1967, when it caused outbreaks of severe hemorrhagic fever in laboratory workers in Belgrade, Yugoslavia, in Frankfurt and in Marburg, West-Germany (1,2,3). These outbreaks were restricted to employees of institutes and factories working in the field of Poliomyelitis vaccine production or safety testing of vaccines (2). There were 26 primary and only 6 secondary cases. Seven out of the primary cases (26,9%) were fatal (3). The common epidemiological denominator of all primary cases was contact to vervet monkeys (*cercopithecus aethiops*), which had been imported to Europe from Uganda. Monkeys from the same shipment had been transported to Belgrade, to Frankfurt and to Marburg (2). Several smaller outbreaks of MBG disease have since 1967 occurred in Africa (5,6).

In August 1976 two outbreaks with severe hemorrhagic disease occurred in Yambuku in the Bumba district in northern Zaire and at the same time in Nzara and Maridi in Southern Sudan (7,8). Viruses with structural homology to MBG virus were isolated from patients in both events and received the names Ebola virus (the Zaire isolate) and Maridi virus (the Sudan isolate) (7,8). 318 cases were observed in Zaire and the case-fatality rate amounted to 88%. In Sudan 228 patients were involved and the fatality rate was 51% (9,10). One case of laboratory infection with Maridi virus has been reported in the context with this outbreak. In 1979 a second epidemic of Maridi virus occurred at Maridi with 34 cases and with a lethality of 22%. (11) The Maridi virus is different from the Ebola (EBO) virus and is now believed to be a viral subtype (12,13).

A third virus of the same group, the Reston (RES) virus, was isolated in 1989 in the USA from Javanese monkeys (*cynomolgus*) which had been imported for biomedical research (14,15). The monkeys suffered from hemorrhagic fever but a second virus - simian hemorrhagic fever virus - was also found during this epidemic.

Virus properties and classification: MBG and EBO virus are the founder members and prototypes of a new virus family, the filoviridae (16). They are rodshaped viruses with a lipid membrane and a helical nucleocapsid (1,7,8). The length of a standard particle is 685 nm in MBG and 805 nm in EBO virus (17). They contain an unsegmented single-stranded RNA genome of anticodon polarity (18,19). Viruses with these genome characteristics are now called mononega-virales. This group includes the rhabdo- and paramyxoviridae and the filoviridae. Filoviruses have seven genes, gene Nr. 4 encodes the message for a glycoprotein (20,21). Comparison of sequence data revealed a 40% homology in the L-Gene with the pneumoviruses (22).

Epidemiology: the natural host and virus reservoir of the known filoviruses is still undetermined. Monkeys, which are believed to have introduced MBG virus to

Europe (1,2) have not been involved in the known African outbreaks (5,6) and they have apparently not been connected with the occurrence of human EBO virus infections (7,8). The Reston virus, on the other hand was introduced to the USA by Javanese monkeys and it has also been detected in monkeys at their place of origin (14). The finding of RES virus in South East Asia is the first evidence that filoviruses are not restricted to the African continent.

Host range and virus transmission: MBG and EBO virus have never been isolated from any wild living animal. Both viruses have been adapted to guinea pigs with a dramatic increase in pathogenicity (1,7,8). Suckling mice are susceptible for EBO but not for MBG infection (23). In experimental studies with monkeys all the filoviruses revealed high pathogenicity (24,25). In contrast to MBG and EBO viruses RES virus has not produced human disease upon inadvertent inoculation although seroconversion did occurr (15).

MBG virus transmission from infected to uninfected guinea pigs is efficient in cage mates but aerogenic transmission to room mates has never been observed (23). In infected monkeys virus transmission to room mates without physical contact was not found to occurr in one study (24) but was seen in other studies (25). The monkeys were shown to excrete 10^6 guinea pig infectious doses per ml of urine (25).

Transmission of MBG virus from monkeys to man was not observed in animal waiters, who cared for the infected animals, but remained restricted to persons performing surgical procedures including removal of kidneys and brain, opening of thoracic and abdominal cavities, autopsies, and taking of blood samples (2). In experimental infection of monkeys a viremia of 10^{10} monkey infectious doses was found (25). Mincing and trypsinization of kidneys and other organs from infected monkeys has produced human infections (2). MBG virus was also shown in monkey kidney cells, which were prepared from infected monkeys (24).

The efficiency of human to human transmission is very low in filovirus infections. There were not more than 6 secondary cases in the 1967 MBG virus outbreak. These could be traced to needle stick inoculations in two cases, unprotected contact with patient blood in two cases, and incoulation with a knife at autopsy in one case (3,26,27)). Only one case of a "private" human to human transmission was found in spouses where the man infected his wife 3 months after convalescence and the virus was isolated from his semen (28,29). One of the patients shared bed with his brother and died few days after hospital admission. The brother had no evidence of virus infection (23).

In the Zaire outbreak of EBO virus infection the virus was distributed in a maternity ambulance and in a hospital by way of injections with reused syringes as well as by contact with blood from bleeding patients. Out of the Yambuku hospital staff 13 out of 17 contracted the disease and died. Up to five generations of human to human transmission were reported to have occurred in EBO virus infections but the spread to family contacts other then spouses or brothers and sisters was low (8,9).

Serological surveys of human sera for filovirus antibody have shown a seroprevalence of up to 50% in several populations of Equatorial Africa (30,31,32). The combination of high seroprevalence and low efficiency of transmission points to the possibility that vectors might be involved in the spread of filovirus infections. Serological evidence of filovirus infection has been found in more than 50% of German patients with imported malaria. Seroconversions against MBG virus were found in cases of fresh malaria and viral RNA was detected in two cases of malaria by PCR (33,34).

The clinical symptoms of filovirus disease are characterized by malaise with fever up to 40^{o}C, headache, myalgia, drowsiness, vomiting, diarrhoea, enanthem, rash, lymphadenopathy and conjunctivitis. Characteristic symptoms of hemorrhagic diathesis occurred in about 30% of the patients and was often indicative of fatal outcome (1,3,26,27,7,8). Fatal cases were associated with pneumonia, pleuritis, and with cardiac and renal failure. Among the laboratory parameters a rise of SGOT but not of SGPT and a drop of white blood cell and thrombocyte counts was a characteristic finding in most cases. Platelet counts of 20 000 cells were found in patients with severe hemorrhagic symptoms (3,26,27).

Patients with a fatal course died at the end of the first week of disease or in the second week with symptoms of severe hemorrhagic shock with black vomiting, melaena and urogenital and with intracranial bleedings (3,26,27).

Therapy: There is no established therapy of filovirus disease. Transfusions of fresh blood might be helpful in cases with hemorrhagic diathesis. In vitro studies have shown that antimalaria drugs can suppress the replication of MBG and EBO virus in culture (23).

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David K. Henderson
The Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda; United States

HIV-1 and the Health-Care Worker - 1993

This presentation will discuss in detail the epidemiologic, clinical, and serologic events surrounding the occurrence of a documented occupational infection in a clinical laboratory worker at the Clinical Center, the hospital at the National Institutes of Health, Betheda, Maryland, USA. The discussion will focus on the factors that have been proposed as possibly contributing to the risk for occupational infection and will address the risk for occupational transmission of HIV-1 in clinical and laboratory health-care settings in the context of information available and relevant to the discussion.
Specifically, this occupational infection will be discussed in the context provided by: population-based epidemiologic studies of the risk for occupational HIV-1 infection in clinical and laboratory workers, current knowledge about occupational HIV-1 infections among clinicians and laboratorians working in healtcare settings, an assessment of the data available from longitudinal or prospective studies attempting to estimate the risk for HIV-1 transmission to health-care providers and laboratory staff following discrete, documented exposures to HIV-1 in the workplace, and a comparison of the risks associated with these exposures to other infectious and noninfectious risks present and prevalent in the healt-care and laboratory worksites.
The presentation will also address prevention strategies for HIV-1 infection in these workplaces, including the use and utility of Universal Precautions, the management of occupational exposures and the rationale for and against the use of zidovudine as post-exposure chemoprophylaxis.

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D. Onions
Dept. of Veterinary Pathology, University of Glasgow, Glasgow, Great Britain

The safety Evaluation of Cell Lines used in Biotechnology: Screening for Adventitious Agents

Introduction

The sources of microbiological contamination in cell systems arises from several sources. Viruses can persist from the original animal from which the cells were derived, an extreme case of this is exhibited by the endogenous retroviruses which are present in the cells as part of their normal genetic makeup. A second and important source of contamination arises from products of animal origin used to grow or store the cells. Although there is an encouraging trend towards the use of defined tissue culture media which do not utilise such materials, most cells have been stored in foetal bovine serum and possibly removed from tissue culture flasks with trypsin of animal origin. A final source of contamination is from the operators or the environment under which the cells are handled. Good manufacturing practice should limit the possiblility of contamination from operators but it does occur.

The use of antibiotic free medium imposes a strict discipline on the operators and enables bacterial contamination to be detected easily. Consequently, while appropriate tests for bacterial and mycotic sterility must be undertaken, most problems arise from mycoplasma and viral contamination. In this symposium the potential problem of mycoplasma contamination has been addressed by Dr Henrich and Dr Diggleman has delt which specific problems associated with retroviruses.

In vivo assays the MAP, and HAP tests

Amongst he most widely used cells in biotechnology are murine hybridoma cells and CHO cells derived from the Chinese hamster. A standard method of detecting a wide range of possible indigenous contaminants are the mouse and hamster antibody production tests (MAP and HAP). In this procedure the test article cells are inoculated into SPF mice or hamsters and the animals followed to determine if an antibody response develops, indicating the presence of the virus.

Twenty-two viruses are listed as potential contaminants of murine cell lines in appendix 1 of CPMP's guidance notes for the production and marketing of murine monoclonal antibodies (CPMP 1989b) and of these 16 are normally detected in standard MAP tests. The remaining viruses, lymphocytic choriomentingitis virus (LCMV), mouse cytomegalovirus, mouse rotavirus (EDIM), thymic virus and lactic dehydrogenase viruses and retroviruses require specific assay systems.

Retrovirus tests must always be conducted and it is usual to ensure freedom from LCMV by an appropriate *in vivo* test.

The significance of LCMV virus as a contaminant has been reinforced by the recent description of infection of animal house workers by LCMV. (Mahy et al 1991). LCMV is an arenavirus that establishes a silent, persistent infection in mice following perinatal transmission whereas transmission to adults causes severe, often fatal lymphocytic choriomeningitis. In the outbreak described by Mahy, the infection was first uncovered when one of the workers in the facility developed meningitis. Subsequently a total of 8 of 82 workers were shown to have serological evidence of infection and cell lines passaged in nude mice were found to be infected with the virus, one line having been infected since 1975.

In general, with the exception of retroviruses, there should be no evidence of virus contamination by any virus. Under no circumstances can cells containing the viruses in group 1, which can cause disease in man, or polyoma virus from group 2, be used for the production of biologicals.

As a supplement to the MAP or HAP tests, *in vivo* challenge of animals and inoculation of embryonated eggs is used to reveal the presence of adventitious agents. At least 5 guinea pigs, 10 adult mice and 10 suckling mice from two litters, are inoculated intramuscularly with test article material. A further 10 adult mice receive an introcranial inoculation of the test article. These procedures are valuable in demonstrating the presence of certain viruses that are difficult to detect in *in vitro* assays. Morbidity and mortality are taken as indicators of infection in the animals and the egg cultures are inspected regularly for gross changes and the contents screened by haemagglutination at the end of the assay.

Assays for bovine viruses and other viruses of animal origin

A significant cause for concern has been the presence of viruses of animal origin as contaminants of cell lines. Most emphasis has been placed on bovine viruses that may be present in serum used to grow or store cells but it should not be forgotten that a range of other animal products, like porcine trypsin, may be used in tissue culture. A complete testing schedule should take account of these non-bovine viruses.

To detect bovine viruses at least two bovine indicator cell lines, susceptible to the major viruses of concern are inoculated with a test article. The detection of viruses depends on two principal properties. First the viruses may be cytopathic, producing a recognisable change in the appearance of the cell sheet. Often particular virus groups produce a characteristic change. For instance herpesviruses often produce syncitia by fusing the membranes of adjacent cells while adenoviruses block the Na^{++} pump so that the cells become characteristically swollen. Some viruses also haemagglutinate erythrocytes or the infected cells may bind i.e. haemadsorb red blood cells. This provides a sensitive indication of the presence of some viruses like parainfluenza viruses that may be minimally cytopathic. However certain viruses require very specific systems to

reveal their presence and amongst these are BVD virus, bovine polyomavirus and the uncharacterised agent of bovine spongiform encephalopathy.

BVD-Virus. The BVD virus is a member of the pestivirus group related to swine-fever virus of pigs and border disease virus of sheep. It is frequently transmitted *in utero* so that calves are born persistently infected with the virus. Usually these strains are non-cytopathic and they will be present in the foetal serum at high titre as, the calf or foetus is tolerant to the virus. (Brownlie et al 1984). BVD is capable of infecting cells of a range of different species (King and Harkness 1975) and as it is not often cytopahtic it is not easily revealed. In order to reliably detect the virus it is necessary to infect a permissive cell system and, after repeated passage of the cells over at least 3 weeks, it may be revealed by immunofluoresence with specific anti-sera. More recently the polymerase chain reaction (PCR) has been used to detect both infected serum batches and infected cell cultures.

The bovine polyomavirus. The bovine polyomavirus is a good example of a virus that has been known to science for some time but has received little attention until recently as it has not been important in veterinary medicine. However, it is an extremely common contaminant of bovine serum, it is possibly a zoonotic virus and it belongs to a family of potentially oncogenic viruses. It is therefore of little surprise to find that it has become the focus of some concern in the biotechnology industry.

Bovine polyomavirus was first isolated from kidneys of newborn calves. Subsequently Parry and his colleagues (Parry et al 1983) detected a polyomavirus in a rhesus monkey kidney cell line used to grow hepatitis A virus. Other primate cell lines including vero cells were shown to harbour a related virus. The high prevalence of antibodies to bovine polyomavirus in the UK cattle population, 62% being positive, indicated that bovine serum was the likely source of these viruses in cell culture. A surprising and worrying feature of the sero-epidemiology of this virus is that while antibody to it was generally absent from the human population, 71% of veterinarians, 50% of cattle farmers and 40% of abattoir workers had detectable antibody that was not cross reactive with human polyomaviruses (Parry and Gardner 1986). This virus must therefore be considered potentially zoonotic the agent has been transmitted to cats through the consumption of infected food (Leggett et al 1990).

Bovine spongiform encephalopathy. BSE must clearly be a matter of concern in evaluating products directly derived from bovine material. However it is not of general concern in products derived from cell culture. First, it is now required to use serum from non-endemic areas for BSE, like New Zealand or the United States of America. Secondly in infected animals the titre of the agent is very high in brain tissue ca.10^9/g whereas in serum it is undetectable (i.e.<10^2 infectious units/g) in the *in vivo* assay used to detect the agent. Moreover, while high titres of the scrapie agent can infect rat neuronal cells in culture, most cells are probably resistant to infection. In general because the assay for scrapie requires a year or more it is not practicable to assay for the agent in cell lines or serum batches. Where necessary a validation of the downstream purification process can be conducted to test its capacity to remove the scrapie agent.

It is now believed that the origin of the disease, first recognised cattle in the United Kingdom in 1986 arose from the consumption of feedstuffs containing sheep protein contaminated with the scrapie agent. In turn it was a change in the treatment of ovine carcasses in the rendering process that allowed the extremely resistant agent of scrapie to get into the food chain (Wilesmith et al 1988). Indeed the scrapie agent is highly resistant to many physical treatments and even the recommended treatments by autoclaving at 134-138°C for 18 min, treating with sodium hydroxide 1N for 1h at 20°C are not guaranteed to abolish infectivity (CPMP 1992). The nature of the agent remains unresolved but is the subject of intense scientific interest. Among the competing hypotheses are an infectious protein, or prion and an unconventional virus with free nucleic acid protected by a large molar excess of the abnormal cellular protein PrP^{Sc}, that is deposited as fibrils in the brains of affected animals (Hope et al 1988).

A further factor of concern is that animals including mice pigs and hamsters can be experimentally infected with the agent by intracerebral inoculation. Moreover in this University we have recently recorded the presence of spongiform encephalopathy in cats. We have not observed this pathology in brain sections in the previous ten years and it must remain a possilility that the agent has been transmitted to cats through the consumption of infected food (Leggett et al 1990).

In Vitro assays for viruses

In addition to the specific assays for bovine assays a general in vitro assay is required to detect viruses particularly those capable of infecting human cells. Usually a test article prepared from the cell line is used to inoculate between 2 to 6 cell lines and the test article cell line itself. The FDA recommend a minimum of 2 cell lines including a human diploid cell line like MRC-5 and a monkey cell line like vero. The CPMP recommend that in addition a cell line from the species of origin of the test cells be included and a bovine cell line. However, as described above bovine viruses are examined in specific assays and it is more usual to widen the range of cells to include a human epithelial cell line like HeLa, that is permissive for adenoviruses, and the RK13 cell line permissive for rubella.

The principal of the assay is that the cells are examined for a total of 28 days with a blind passage of negative cells at 14 days. In addition to observing for cytopathic effect the cell system is examined for haemadsorbing and haemagglutinating viruses with human O, guinea-pig and chicken erythrocytes. It is of course essential to have both negative controls and positive control cultures infected with viruses that will display haemagglutination, haemadsorption and a cytopathic effect. One cautionary point needs to be emphasised. While many tissue culture adapted strains grow well in these systems this is not always true for primary isolates. Experience is required to detect the subtle changes that might be associated with such wild type viruses.

Retrovirus assays

Retroviruses pose one of the most important challenges in developing products from cell systems as these viruses are ubiquitous and associated with life-threatening conditions including cancer and immunosuppression. However, by conducting an integrated approach to testing, at each stage of the production cycle, the problem of retrovirus contamination can be contained.

Many of the pathogenic properties of these viruses are associated with their replication cycle (Jarrett and Onions 1992). The viruses are enclosed in an outer envelope containing viral glycoproteins. These glycoproteins bind to receptors on cells and, in part, define the host range of the viruses. Once bound the genomic RNA of the virus is liberated into the cytoplasm where it is transcribed into double stranded DNA by a virion encoded enzyme, reverse transcriptase. The DNA copy or provirus migrates to the nucleus where it is covalently integrated into chromosomal DNA. At this stage the virus may remain as a latent infection or it may be transcribed into new mRNA and genomic RNA. New virus particles are assembled at the cell surface and are budded from the cytoplam. This process is not cytopathic for the *oncovirus* sub-group of retroviruses so they continue to shed virus while the cells replicate and function normally.

In probably all vertebrate species some retroviruses have succeeded in infecting the germ line so that provirus are passed from generation to generation as conventional genes. Usually the expression of these viruses is tightly repressed in the individual but tissue culture cells may spontaneously begin to produce them. In addition to these endogenous viruses, many species can be infected by exogenous retroviruses transmitted from animal to animal like conventional viruses. In some cases these can undergo recombination with endogenous retrovirus elements to generate new subgroups with altered host cell ranges. Some members of the oncovirus group, including the murine leukaemia viruses (MuLV) can recombine with cellular transforming genes (oncogenes) so that the progeny virions carry these within their genome. This forms one of the principal methods by which these viruses transform cells.

Retroviruses are sub-divided into the *oncoviruses* like MuLV the lentiviruses which includes HIV and the *spumaviruses* that do not have any clear disease associations. The oncoviruses are further divided by the organisation of their genomes and by their appearance in the electron microscope so that MuLV is described as a type-C virus because its core is formed during the budding process.

The murine leukaemia viruses exemplify the complexity of these viruses. Cells may release a number of different viruses defined by their host range. Ecotropic viruses infect only murine cells whereas xenotropic viruses released by most murine hybridomas infect cells of many species but not murine cells. Murine xenotropic viruses replicate efficiently in mink cells but less efficiently in human cells and indeed the xenotropic viruses from murine hybridomas do not usually replicate in human cells. However, the situation can be more complex as ecotropic viruses can undergo recombination with other endogenous sequences to generate polytropic viruses capable of replicating in murine and non-murine cells. These polytropic viruses are often the viruses that indicate leukaemia in laboratory mice. Amphotropic murine viruses also replicate in murine and non-murine cells but their

origin is from wild mice. However, they have been used as the basis of retrovirus packaging lines now being used in gene therapy protocols.

A screening system for retroviruses must take account of this complexity of host ranges and different assays are required to detect these different virus groups (Onions and Lees 1991). For murine cells the ability of ecotropic viruses to produce syncitia on rat XC cells froms the basis of a quantitative assay, one XC plaque being formed by one infectious virus particle. Similarly xenotropic and amphotropic viruses can be detected by their ability to rescue defective oncogene containing viruses from so called S+L-cells. The oncogene containing virus rescued by these defective viruses transforms adjacent cells producing a focus of rounded cells; again one focus is induced by one infectious virus so that the assays are quantitative. Polytropic viruses may be positive in the XC assay but they also produce a degenerative cytopathic effect on mink cells, so that by a combination of XC and S+L-assays one can detect the retroviruses of concern from murine cells.

In these assays it is usual to plate the supernatant from the test cell line directly onto the XC or S+L-, which constitutes a direct assay. However if the virus is present at low titre it may be missed for two reasons. First if the titre is 1000 infectious units per litre then if 1 ml is assayed the virus will be missed on 37% of occasions simply because of the distribution of the virus in the sample as given by the Poisson distribution. Moreover while one virus particle produces one plaque or focus not every virus "hit" does this. Consequently direct assays become inaccurate at titres below 10^1 ml. One method of circumventing this problem is to conduct extended assays in which the indicator cells are passaged 5 times over a 3 week period, which allows the virus infection to spread throughout the culture system. These assays are quantal *i.e.* positive or negative, rather than quantitative assays, but they are more sensitive than their direct equivalents. A further level of sensitivity can be achieved by co-culturing the test cells with the indicator cell lines.

In devising a retrovirus screening assay at least three independent methods are used:

- Transmission electron microscopy is used as general screening technique on cells for adventitious agents but it is a valuable method of detecting type-C retrovirus particles and non-infectious retrovirus like elements called intraciternal type-A particles. (Other type A particles can be the pre-formed cores of type B or D viruses.)
- When testing a number of clones for cell banking purposes it may be appropriate to use direct assays as an initial screen but extended or preferably co-cultivation assays should be used on apparently negative clones to be sure of their status.
- The reverse transcriptase assay provides a broad screening system for many different retroviruses. It does not detect infectious virus but detects the presence of extra-cellular virus particles containing this enzyme. The distinction is important as for instance, CHO cells may release reverse transcriptase containing particles but these are defective containing deletions within their genomes. However one should be cautious in always assuming that CHO viruses will be non-infectious as recombination is frequent in retroviruses and

repair of such defects can occur. While reverse transcriptase assays are simple to conduct their control and interpretation requires care. Contaminating cellular polymerases, particularly γ DNA polymerase, can use the synthetic RNA templates used to monitor reverse transcriptase activity. For this reason it is essential to include a synthetic DNA template to measure DNA polymerase contamination. Retroviruses also differ in their cation requirements for reverse transcriptase activity and the assay should be conducted separately with Mn^{++} and Mg^{++} as the divalent cation.

Whenever evidence for retrovirus production is produced by any of these systems the cell line should be co-cultured with human cells to determine if the virus is capable of infecting human cells.

Although some cell lines might initially be negative for retrovirus production they may, at subsequent time points become positive. The stability of non-producer cells can be tested by treating them with thymidine analogues like iododeoxyuridine.

In the case of cell lines other than murine and CHO cells it is usually necessary to devise specific retrovirus assays. For instance, in human cells it is essential to screen for the human leukaemia retroviruses, HTLV-I & II, and the lentiviruses HIV I & II although these are not likely to be contaminants of non-lymphoid cells (Onions and Lees 1991b). These viruses are exogenous so that it is possible to use DNA hybridisation and PCR approaches as a supplement to culture techniques.

Retrovirus contaminants can sometimes go unnoticed for long periods. For instance many stocks of the marmoset cell line B598 used to produce EBV for preparing human hybridomas are also contaminated with a squirrel monkey type-D retrovirus (SMRV) (Popovic et al 1982).

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J. Schüpbach, H. Pyra, J. Böni
Swiss National Center for Retroviruses, Universitiy of Zurich, Zurich, Schweiz

Detection and quantitation of filterable particle-associated reverse transcriptase (RT) in retroviral infections by a new test of 10^7x increased sensitivity

The role of HTLVs and HIVs in human disease has been well established. Involvement of additional retroviruses (RV) in some other diseases is likely. A universal screening test for RV as sensitive as PCR, but independent of viral sequence, would be of great value, also with respect to the detection of adventitious infectious agents in cell cultures, blood products, vaccines or other biological materials used for human treatment.

A novel test for RT, PRODUCT-ENHANCED REVERSE TRANSCRIPTASE (PERT) assay, was developed and used for the detection of filterable, particle-associated RT in plasma samples from patients with HIV-1, HIC-2, or HTLV-1 infection, more than 200 healthy blood donors, and animals infected with different RV. In the PERT assay, genomic RNA of bacteriophage MS2 serves as the template for the RT-mediated cDNA synthesis. A fragment of the cDNA is then selectively amplified by PCR and the amplification product analyzed by Southern blot hybridization or enzyme immunoassay.

The PERT assay was 10^7 x more senitive than conventional RT test and, like RNA-PCR, detected as few as 10 to 100 virions. All of 30 HIV-1 plasma were positive; HIV-2, HTLV-1, FeLV, FIV, and MuLV RT were also detected. Two blood donors were positive, one with a borderline-positive HIV screening test and antibodies to HIV-1 p24, but no seroconversion, for 3 y, the other with slightly abnormal liver markers, but negative hepatitis virus serology, for 3 y. Both were negative for HIV or HTLV by DNA and RNA PCR, but PERT activity coresponded to 10^4 - 10^5 particles/ml plasma. In cell cultrures established from HIV-1 infected patients, the PERT assay identified the presence of the virus even when analysis of supernatants by a very sensitive antigen p24 detection procedure was negative.

The PERT assay is a universal ultrasensitive screening test for RV or other RT-associated agents. It will be improtant for diagnosis, the safety of the blood supply, and recognition of unknown, replication-competent retroviruses in vitro and in vivo.

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Dr. Hannelore Willkommen
Paul-Ehrlich-Institut, Langen, FRG

EC regulatory framework for the safety of mammalian cell culture products

The EC regulatory framework covering cell culture products consists of different dokuments:

1. Production and quality control of medicinal products derived by recombinant DNA technology (June 1987, revision under discussion)
2. Production and quality control of monoclonal antibodies of murine origin (June 1987)
3. Production and quality control of cytokine products derived by biotechnological processes (February 1990)
4. Production and quality control of human monoclonal antibodies (July 1990)
5. Validation of virus removal and inactivation procedures (February 1991)
6. Minimizing the risk of transmission of agents causing spongiform encephalopathies via medicinal products (December 1991)

The aspects of the testing and control of virus safety of medicinal products produced by menas of mammalian cell lines are discussed on the basis of these documents. The recommendations are compared with the "Points to Consider in the characterization of cell lines used to produce biologicals", the regulation framework of the FDA.

The rules governing medicinal products in the European Community

Volume I. The rules governing medicinal products for human use in the European Community; Catalogue number CO-71-91 631-EN-C

Volume II. Notice to applicants for marketing authorizations for medicinal products for human use in the Member States of the European Community (Second edition); Catalogue number CB-55-89-293-EN-C

Volume III. Guidelines on the quality, safety and efficacy of medicinal products for human use; Catalogue numberCB-55-89-843-EN-C
Addendum I (July 1990) CB-59-90-936-EN-C
Addendum II (May 1992) CB-75-92-558-EN-C

Volume IV Good Manufacturing Practice for medicinal products
Catalogue number CO-71-91-760-EN-C

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