Additional information is provided below on a number of commonly used viral systems. It is not intended as an exhaustive guidance on a particular system nor to provide a substitute for a thorough risk assessment, but rather to draw users attention to particular points that should be considered during the risk assessment.

ADENOVIRUSES

Considerations relating to the vector

1. Human adenovirus is a double-stranded DNA virus comprising over 40 serotypes all of which are categorised as hazard group 2 biological agents. They vary in their pathogenicity; some serotypes (Ad12) produce sub-clinical infections; some (Ad5) are associated with mild respiratory infections in children whilst others (Ad4 and Ad7) are associated with acute respiratory disease in adults. The normal route of infection in the respiratory tract is thought to be via aerosols. Adenovirus are also implicated in conjunctivitis and gastro-enteritis (Ad40, for example, causes infantile diarrhoea). Wild-type human adenovirus is a hazard group 2 biological agent and as such ACDP/ACGM containment level 2 should be adopted as a minimum.

2. Immunity to adenovirus infections is thought to be life-long following primary infection, although latent infection of tonsil and adenoid tissues is a frequent occurrence following childhood infection by Ad1, 2 or 5. The precise mechanism of latency is unknown, but free virus is only rarely detected in lymphoid tissue and is present in <1 in $10^7$ cells. Reactivation of latent Ad5 can lead to serious complications in immuno-compromised individuals and it has been isolated at high frequency from patients with AIDS. In addition to the occurrence of latency it has also been shown that Ad2/5 E1a sequences can be found in respiratory epithelium samples from approximately 20% of normal healthy adults. However, these sequences are thought to exist in the form of fragments of the genome that are a remnant of previous exposure rather than as intact genomes that might be capable of reactivation.

3. Human serotypes do not normally infect other animals and there are few reports of virus replication following inoculation of experimental animals. The cotton rat, a species not indigenous to the UK, provides an animal model of infection. Adenoviruses can be divided into three major groups based on their ability to produce tumour in new-born rodents. Group A viruses (e.g. Ad12) are highly oncogenic; Group B viruses (e.g. Ad7) are weakly oncogenic and Group C viruses (e.g. Ad5) are non-oncogenic. However, all human adenoviruses, including Group C, transform rat cells in culture. Despite this, there is no evidence of any association between adenoviruses and human cancer.

4. Adenovirus binds initially to target cells through the fibre protein, however the cellular receptors have not been unambiguously identified. Subsequent cell entry involves interaction between the capsid penton proteins and integrins on the target cell; different serotypes may use different cellular receptors and integrins. Adenoviruses have efficient mechanisms for internalisation to cell cytoplasm from endosomes. Inside the cell, the early genes including the E1a and E1b transforming genes are expressed, leading to replication of the ~36 kb viral genome as a linear episome, and expression of the late genes which encode capsid proteins. Between ~100 and $10^5$ viral particles may be produced per cell, in a lytic cycle lasting 24-48 hours depending on the virus. Adenoviruses have no mechanism for cytolysis and so in culture, most viral particles remain cell-associated. Sufficient particles are released however, or otherwise gain access to infect neighbouring cells. After several rounds of replication, in confluent cell cultures visible plaques of infected cells are produced, in which the cells show typical cytopathic effects (rounding up of the cytoplasm and clumping into grape-like clusters).

Disabled adenovirus vector
5. The lytic cycle of Ad5 is divided into the early and late phases. The early genes are expressed from four regions of the genome. The two transcription units of the early region 1 (E1a and E1b) are responsible for cell transformation and tumourigenicity. Both alter transcriptional regulation during infection and transform by directly interacting with cell proteins involved in transcription and cell cycle regulation (e.g. p53 tumour suppressor protein).

6. The majority of defective Ad5 vectors have a deletion removing most of the E1 region preventing expression of E1a and E1b genes. The inverted terminal repeat (1 - 103 bp) and packaging signals (194 - 358 bp) must be retained for viability. Such viruses are unable to replicate except in complementing cell lines such as 293\(^1\) (a human embryonic kidney cell line which expresses the left 11% of the Ad5 genome), and foreign genes can be inserted in place of the deleted E1 genes. Adenovirus cannot efficiently package genomes longer than ~105% of the wild type length, so a 2.9 kb deletion in E1 allows insertion of up to 4.7 - 4.9 kb. Vectors have been produced with additional deletions within the viral genome, removing sequences from the E3 or E4 regions. Deletion of the E3 region (which is non-essential for in vitro growth) allows inserts of up to approximately 8 kb to be cloned. Alternatively, deletions in parts of the E4 region can be made without affecting normal growth, permitting an extra 1.8 kb insertion.

7. Genetically modified viruses are usually produced by manipulation of partial viral genomes in bacterial plasmids; co-transfection of overlapping plasmids into 293 cells allows the generation of complete genomes by homologous recombination. Alternative strategies involve in vitro ligation of linearised plasmid DNA to generate the full length genome, prior to transfection of 293 cells.

8. The replication defective Ad vectors have no mechanism for long term maintenance in cells; expression in the lining of the respiratory epithelium declines with time and is limited to ~2 months. In the absence of any significant episomal replication of E1a deleted Ad5 in normal human cells, long term maintenance requires integration in to the host chromosome. This can occur at a frequency of about 1 per 10\(^6\) pfu in exponentially growing cultures of primary human cells.

9. Replication-defective E1a vector can be considered to be unlikely to cause disease for the purposes of any re-categorisation into a biological agent hazard group. It is, however, important to properly take account of the nature of the inserted gene and the characteristics of the final recombinant virus.

10. An E3 deletion will reduce the likelihood of a GMM causing harm as a consequence of making the virus less able to establish and maintain an infection within the cells of an infected individual i.e. the fitness of the virus as an infective agent will be reduced. It should be noted, however, that there is little evidence that the pathogenicity of an E3-deleted virus, in any cells which do become infected, will be reduced. Indeed, if anything, there may be an increased inflammatory response.

**Effects of the inserted sequences**

11. The risk assessment should take into consideration the likely level of expression of an inserted gene, as well as the likely biological activity of the expressed protein. The level of expression will depend both on the cell type, and the regulatory sequences used to control transcription of the inserted gene. For example, use of the enhancer and promoter from the immediate early transcription unit of cytomegalovirus is expected to lead to high level expression in a wide variety of cell types; the promoter from a cellular “housekeeping gene” might lead to a lower level of expression in a wide variety of cells, whereas control sequences from the insulin gene might be expected to restrict expression to β-cells of the pancreatic islets. However, it is always possible that enhancers within the adenoviral vector, in particular the E1 enhancer which overlaps with the packaging signals, could lead to a broader tissue-specificity than expected. The potential biological consequences of a certain level of expression will depend upon the protein, but a potent toxin would clearly have more potential for harm than a simple marker such as luciferase or β-galactosidase.

**Effect of genetic modification on phenotypic characteristics of adenovirus**

a) **Tissue tropism:** Adenoviruses have the potential to infect a wide variety of cell types, although in terms of their natural pathogenicity, they may be grouped into those

\(^1\) The 293 packaging line is increasingly being replaced by those such as PER.C6 which will not produce replication competent adenovirus

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associated with respiratory or enteric disease. Residual "latent" virus can also be associated with lymphoid tissue. Modification of the virus (e.g. to the fibre protein gene) might alter its tissue tropism. As a result, additional tissues might become susceptible to a modified virus.

b) Altered infectivity and pathogenicity: Either the vectors themselves, or inserted genes, might have an altered infectivity or pathogenicity relative to the wild type virus. For example, Ad5 vectors with E3 deletions have increased pathogenicity at least in the cotton rat model; this may relate to the role of the E3 19K protein in inhibiting the translocation of MHC molecules to the cell surface. Deletion of E3 is expected to result in greater presentation of viral antigens to the immune system than in a normal adenoviral infection, resulting in a greater inflammatory response (which might, however, accelerate the clearance of the infected cells). Insertion of other genes, such as cytokines, might also be expected to have some influence on the interaction of the virus with the immune system.

c) Recombination and complementation. There are three or more possibilities for recombination and complementation in E1a Ad5. Homologous recombination between E1a- Ad and wild-type (WT) virus (or viral sequences in the 293 cell lines) may occur at low frequency, but in some cases the packaging limits of Ad would tend to delete the transgene or make such a recombinant unviable. It has also been shown that recombination only occurs between serotypes within a subgenus and not between subgenera. Co-replication between E1a deletants and WT could also occur due to transcomplementation. This requires co-infection of both viruses and has so far only been demonstrated at high multiplicities of infection (moi). Some cell types have been shown to complement E1a at high moi. These include HeLa cells, where the effect is thought to be due to the endogenous human papillomavirus (HPV) 18 E7 gene product in such cells. Certain other viruses, such as Epstein-Barr virus, have been shown to complement E1a mutant Ad and this possibility should be considered.

d) Vaccination and anti-viral drugs. Vaccination or anti-viral drugs are not available, although vaccines against Ad4 and 7 have been used in US military recruits. In normal healthy humans, immunity following Ad infection is thought to be life-long.

Selection of laboratory containment and control measures.

12. Adenovirus is infectious by the aerosol or droplet route, even when rendered replication defective. In addition, in view of its relatively robust nature, its choice as a vector for cloning harmful genes requires a rigorous approach to risk assessment. In cases, where a potentially hazardous gene has been inserted, it is recommended on the basis of safety considerations that work should be undertaken in a microbiological safety cabinet. However, there are some types of recombinant adenoviruses which could, on the basis of safety considerations, be handled on the open bench, even if in practice class II cabinets are used to protect the infected cell lines from contamination. Such recombinant adenoviruses include replication defective vectors in which either marker genes (e.g., GFP or β-galactosidase) or certain therapeutic genes such as those coding for pro-drug activation enzymes.

Risk assessment for environmental protection

13. Adenoviruses are non-enveloped, relatively resistant to desiccation stress and can survive in aerosols. There is no evidence that human Ad serotypes can naturally infect animals, and replication is very limited in mouse cells, for example. However, replication has been shown to occur in the lungs of experimentally infected cotton rats administered a high doses of virus.

RETROVIRUSES

Considerations relating to the vector

14. Retrovirus vectors are particularly efficient systems for the introduction of genes into dividing cells. The virion contains two copies of an RNA genome which is reverse transcribed and
integrated as a DNA provirus into the chromosomal DNA of the target cell. The host range of the viruses is dependent on a number of factors including the specificity of virion envelope glycoproteins which serve as cellular receptors and on other structural proteins which influence post entry blocks to integration. Retroviruses may be subdivided as follows:

**Oncoviruses not containing transforming sequences e.g. viruses of the murine (MuLV), feline (FeLV) and avian (ALV) group**

15. It is from this group that most retroviral vectors have been derived. The proviruses of the simple oncoviruses contain 3 main gene groups; the gag gene encoding the internal structural genes, the pol gene encoding the reverse transcriptase and integrase functions and the env gene coding for the envelope glycoproteins. Transcription of the provirus is regulated by sequences in the 5’ long terminal repeat (LTR) which contains enhancers and a promoter. The underlying principle of retrovirus vector systems is that sequences necessary for packing viral RNA can be identified. In all retroviruses, the major, but not necessarily the only, determinant of RNA packaging is a sequence lying between the 5’ end of the RNA transcript and gag.

**Oncoviruses containing oncogenic sequences derived by recombination with cellular sequences.**

For example, Rous sarcoma virus is a replication competent virus containing the v-src oncogene.

**Oncoviruses belonging to the Human T-Cell lymphotropic virus (HTLV) and bovine leukaemia virus (BLV) sub-group.**

These viruses have complex genomes whose gene products have trans-activating and transforming properties.

**Lentiviruses**

A group which include immunosuppressive viruses like HIV and viruses associated with inflammatory and degenerative diseases of animals. Lentiviruses have complex genomes with some gene products possessing transactivating functions.

**Spumaviruses**

Others including mammary tumour virus.

**Retroviruses as insertional mutagens**

16. Retroviruses of the MuLV, FeLV and ALV groups can act as insertional mutagens. The principal mode of action is through transcriptional activation of genes adjacent to the site of insertion, a process which is dependent on the enhancers or the enhancer and promoter within the viral long terminal repeat. Active insertional mutagenesis of this form may be attenuated by alterations to the viral enhancer. Self inactivating (SIN) vectors have deletions in the parental U3 region containing the enhancer. The progeny SIN vector-proviruses, integrated into the target cell, lack 5’ and 3’ U3 regions. As such these vectors are not easily mobilisable by superinfection with wild type virus, nor are they capable of insertional mutagenesis by the process described above. A limitation of these vectors is the low titres obtained from most packaging lines.

**Disabled retrovirus vectors**

17. Disabled vectors have been derived from some of the above types of retrovirus. Most replication defective systems consist of two components, a packaging cell line and the vector. The packaging cell line contains all the structural genes but has a deleted packaging sequence so that the cell line releases virus particles lacking a normal viral RNA genome. Transfection of these packaging cells with a defective retrovirus vector, containing a packaging sequence, results in incorporation of the vector RNA into virus particles. These virions may infect cells and integrate a DNA copy of the vector genome into the target cell but are incapable of further replication.

18. Transfection of a vector into a simple packaging line described above results in the generation of replication competent retrovirus (RCR) through recombination. Wild type virus can even be found in harvests made after transient transfections of this type of packaging cell line and consequently efforts have been directed at improving their safety. Second generation ones like PA317 are an improvement in that, in addition to deletion of the packaging sequence, the 3’ LTR is also deleted so that two recombinations are necessary to generate a wild type virus. Nevertheless, replication competent virus can be generated in these cells.

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19. Third generation packaging lines contain the packaging construct in two components, significantly reducing the frequency of recombination between vector and packaging sequences. These two component packaging lines should be used where possible.

20. It is good practice to demonstrate the absence of RCRs in vector stocks and mandatory where the level of containment proposed assumes that RCRs are not present. For most purposes direct plating of 5% of the vector stock supernatant onto mitotically active indicator cells (e.g. feline PG4 cells for amphotropic MuLV, or XC cells for ecotropic MuLV) is adequate. However, if the insert is a harmful sequence then more rigorous testing methods may be necessary. One way of ensuring that no RCRs would be undertaken three to five passages of at least 5% of the vector stock on permissive cells followed by virus detection using two independent methods. Special consideration is required when assaying for RCRs in vectors intended for use in gene therapy. In this case the assays should be conducted by a competent laboratory operating under good laboratory practice.

**Containment level for retroviruses**

21. For replication defective viruses the specificity of the envelope glycoproteins of the vector virus is a major consideration in assigning the appropriate level of containment. This specificity is determined by the sequences expressed within the packaging cell line. Viruses are frequently classified into ecotropic, xenotropic and amphotropic strains.

22. Ecotropic viruses replicate in the cells of the host species and sometimes in those of closely related species. Xenotropic viruses are endogenous viruses i.e. genetically acquired viruses, that may be expressed from cells of a given animal but are unable to infect cells of that species. Xenotropic viruses may infect cells of many other species with varying efficiencies.

23. Amphotropic viruses are able to infect the cells of their host and the cells of other species. Murine amphotropic viruses were originally derived from the exogenous viruses of wild mice and form the basis of the most widely used packaging cell lines that release virions capable of infecting human cells.

24. The majority of retrovirus vector systems have been based on the oncoviruses of the murine (MuLV), feline (FeLV) and avian (ALV) groups of retroviruses. These can be assigned to ACGM containment level 1 on the basis of their inherent properties. However, this minimum containment level may need to be increased, depending on the nature of the inserted genes, the type of packaging cell lines and the properties of the final virus.

25. Vectors based on complex oncoviruses like the Bovine Leukaemia Virus (BLV) group and those based on non-primate lentiviruses require special consideration as the parental viruses contain sequences with transactivating, transforming or other undesirable features.

26. Work employing GMMs or vectors based on Human T-cell lymphotropic virus (HTLV) and primate lentiviruses (all of which are Hazard Group 3 biological agents) requires careful assessment. They should generally be at ACGM level 3 containment (i.e. Class 3), unless there is a clear justification for using a lower level of containment. There are now several lentiviral vectors in use which may be used at a lower level of containment. Where new viral vectors are developed that are based on lentiviruses, approval should be sought to handle them at a lower level than that given in the Official list of Biological Agents, or other Classification lists.

**Infection of humans working with retrovirus**

27. The major risks of working with retrovirus vectors are associated with needle stick injury as might occur during animal inoculation, exposure to open wounds and aerosolisation of high titre virus stocks.

**Effects of the inserted sequences**

28. In the discussion below it is assumed that the insert within the vector is capable of expression within target cells. In a full risk assessment, attention should be paid to the nature of the insert and the control of its expression. The definition of retrovirus vector as used in this context is the virion and not the proviral DNA used to transfect the packaging cell lines.

29. In most instances, replication defective ecotropic non-primate vectors containing an insert unlikely to be harmful in the target species can be handled at ACGM level 1 containment. Some
consideration of increased containment and control measures may be needed if the product is harmful in the host species. At the very least, this should include consideration of waste disposal methods and some consideration should be given to the need for testing for the presence of replication competent virus.

30. In the case of amphotropic replication defective vectors, access to human cells is a possibility but dissemination of the vector should not occur. In most instances, an amphotropic vector containing a non-harmful insert should not need additional containment and control measures above those indicated above. Where the vector contains a sequence which may be harmful if delivered to the target tissue (e.g. Interleukin-2), it will be necessary to consider additional containment measures, ACGM level 2 should be used as a minimum. Amphotropic vectors containing functional oncogenes should be contained at ACGM level 2. In both cases, attention should be given to the possible need for testing to detect RCV.

Effects of the inserted sequences on the characteristics of the retrovirus

- **Vector inserts encoding viral glycoprotein**

  Consideration should be given to vectors expressing viral glycoproteins or other cell binding ligands which might alter the host range of the vector. For instance the envelope glycoprotein of vesicular stomatitis virus can be incorporated into ecotropic MuLV and will widen the host range of the virions. However, only a limited number of envelope proteins will form pseudotypes in this way.

- **Incorporation of endogenous retroviral elements**

  Packaging cell lines may express retroviral and retroviral related sequences that can be incorporated into the virions released from the packaging line. Particular consideration should be given to this factor if new packaging lines are being developed.

Recombination and pseudotype formation

31. Attention should be paid to alterations in host range that can occur through recombination or pseudotype formation. If a packaging cell line is capable of expressing endogenous retroviral sequences or is infected by wild type virus viruses, pseudotype particles containing the vector genome within the envelope of endogenous or superinfecting virus may be produced.

32. Recombination is of particular concern in packaging cell lines and in animal experiments. Infection of a mouse with the combination of a vector and replication competent ecotropic helper virus can result in recombination with endogenous sequences. The resulting polytropic viruses have an expanded host range. Recombination between vector sequences and endogenous (genetically inherited) sequences is of the order of $10^{-2}$ to $10^{-4}$ per replication cycle where there is some sequence homology between part of the sequences.

Risk assessment for environmental protection

33. Retroviruses require close contact for their transmission and their survival in the general environment is poor. Members of the MuLV/FeLV oncovirus group frequently require high titres of virus to establish persistent infections in immunologically competent animals so that the risk of harm to the environment associated with accidental release of vectors is generally low. Special consideration should be given to the use of vectors in domestic animals or in species whose virology is not well characterised and the advice of MAFF (or devolved equivalents) and HSE sought if necessary. Attention should be paid to the possibility that the vector could be mobilised by a naturally occurring retrovirus in the species. For instance, an ALV vector might be assessed for work at containment level 1 in the laboratory but when introduced into birds that contain wild type ALV, level 2 animal containment might be appropriate.

34. Experiments in mice, using retrovirus vectors mixed with helper replication competent virus should be based on ecotropic vectors if possible. Because of the problems associated with retrovirus recombination in mice leading to polytropic virus production, the inoculated mice and viruses isolated from these mice should be handled at ACGM containment level 2, if the insert is a harmful sequence.

**ALPHAVIRUSES**

Considerations relating to the vector

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35. Alphaviruses comprise several arthropod-borne viruses in the family Togaviridae. Alphaviruses are enzootic, naturally infecting and replicating in mosquitoes as well as other animal species including birds. Humans infected with Hazard Group 2 alphaviruses (e.g. Semliki Forest [SFV], Sindbis [SIN] or Ross River virus) may develop mild symptoms, but recovery is usually uncomplicated and complete. On the other hand, Venezuelan, Eastern and Western equine encephalitis virus are Hazard Group 3 viruses and may produce epidemics of encephalitis in horses or even humans with high mortality rates. Further guidance on individual alphaviruses can be found in the International Catalogue of Arboviruses. The following guidance is based on SFV (and the similar SIN) which are more likely to be used as vectors for genetic modification.

36. Infection of humans or animals by the aerosol route is considered unlikely unless very high concentrations of virus are used to generate the aerosols. The risk of human infection is low if the virus preparation is handled in an appropriate safety cabinet and not injected accidentally. Natural infections only occur when infected mosquitoes take a blood meal from viraemic vertebrate hosts. The risk of natural spread of these viruses (if replication occurs) from one human to another, or from an infected animal to a human, is minimal in the absence of an available competent mosquito vector. However, it is possible that blood to blood contact or infected animal bites may carry a risk of transmission. In general, adult laboratory animals are not susceptible to infection by SIN or SFV although some strains may cause encephalitis in 3 week old mice if the virus is inoculated intracerebrally.

37. The alphaviruses have a single-stranded, positive sense RNA genome which is enclosed in a capsid protein. Two glycoproteins, E1 and E2, are incorporated into the membrane that envelopes the capsid. The virus forms two polyprotein products, one translated from the viral 5’ end sequence, the other from transcription of a subgenomic mRNA corresponding to the 3’ third of the genome, from where the encoded proteins are processed in infected cells to their mature forms and are incorporated into the infectious virus particles.

### Disabled or attenuated alphavirus vector systems

38. Disabled derivatives of SFV have been produced which ensure that the recombinant virus undergoes only one cycle of infection. In vitro transcription of plasmid clones generates a packaging-competent replicon RNA which also encodes the foreign gene(s) and a packaging-incompetent DI helper RNA that encodes the virus structural proteins. These RNAs are co-transfected into permissive cells to generate recombinant, infectious virus capable of only a single replicative cycle. Although the replicon RNA persists in infected cells, no virus can be produced and the extent of the infection is limited to those cells initially exposed to virus. However, replication competent virus (RCV) may be produced by recombination in the packaging cell lines (thought to be due to replicate strand switching between replicon and helper RNAs). A further modification to this system involves the use of strains with a mutation in the p62 spike protein and packaged viruses require in vitro treatment with chymotrypsin before they are able to infect susceptible cell lines.

39. Recognising that either type of disabled derivative can produce RCV, any decision to assign these disabled vectors to ACGM level 1 must be taken at a local level in consultation with the GMSC. The appropriate containment for the final modified virus will depend on the risk assessment considerations below.

40. In a further system (which is not disabled), a full length cDNA copy of the viral RNA is modified to contain a second internal subgenomic RNA promoter positioned downstream of the internal RNA promoter which expresses the structural proteins. Heterologous gene sequences are inserted immediately downstream of the second promoter. This cDNA plasmid template is transcribed in vitro and when the resultant RNA is transfected into susceptible cells a fully infectious alphavirus that expresses the heterologous gene(s) is produced. Recombinant infectious clones using such vectors are generally less stable, losing their inserts on repeated passage mainly due to the lack of editing function in the RNA dependent RNA polymerase.

### Effects of the inserted sequences

41. It should be possible to predict, based on studies in other host-vector systems, whether or not the expression products from SFV

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2 Obtainable from Dr N Karabatsos, Division for Vector-Borne Viral Diseases, Center for Infectious Disease, CDC, Fort Collins, Colorado 80522, USA.

3 Berglund et al. (1993). Biotechnology 11, pp916-920

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recombinant viruses will produce pharmacological or physiological effects on vertebrates or invertebrate cells. The introduction of a known toxin gene would clearly need an assessment of the risks associated with the toxin in the context of the SFV virus recombinant as a delivery system.

**Effect of genetic modification on phenotypic characteristics of alphavirus**

**Tissue tropism**

42. If insertion of an envelope glycoprotein gene from a virus in a different family is intended the assessment should consider the possibility that the gene product might be incorporated into the virus envelope. In general, incorporation is considered to be unlikely because of incompatibility problems (including structural differences in virus envelopes, differing sites and processes involved in virus maturation). If, however, the gene was derived from a related virus then the possibility of incorporation would be high. In assessing risks with viruses modified in such ways, it may also be necessary to consider whether or not there is evidence of pseudotype formation between the parental viruses.

**Altered infectivity and pathogenicity**

43. There are no known vectors for SFV in the UK and the risk of virus transmission to wildlife is considered negligible. Nevertheless, any risk assessment should consider the possibility of altered infectivity where expressed gene products may be incorporated into the virion. If a protein to be expressed is known to have an effect on the immune system this should be taken into account in the assessment.

**Recombination and complementation**

44. With the disabled SFV system described above, the risk of spread through humans or animals in the environment is lower than that with the full length infectious virus, since the virus can only undergo one cycle of replication. Nevertheless, there is a small risk that fully infectious virus will be generated by recombination events during RNA replication immediately following transfection and this must be considered in the overall assessment.

**Vaccination and anti-viral drugs**

45. No control agents or vaccines for SFV infections are available. However where the expressed proteins are derived from other viruses for which vaccination is possible, staff could be vaccinated against the original virus. For example, vaccinations are available for a number of flaviviruses including, tick-borne encephalitis (related to louping ill virus), yellow fever, and Japanese encephalitis virus.

**Risk assessment for environmental protection**

- route of transmission and the risk of spread of the recombinant virus

46. The recognised route of transmission of SFV virus is via infected mosquitoes as they take a blood meal from a vertebrate host. SIN and SFV were originally isolated from Culex and Aedes mosquitoes respectively but they have subsequently been shown to infect a number of other species of mosquitoes in the tropics and sub-tropics. Such arthropods are believed to be the only natural means of replicating and transmitting the viruses to susceptible vertebrates. In mosquito-free laboratories and taking into consideration the lack of known vectors for SFV in the UK, the risk of transmission to mosquitoes and vertebrates (wildlife) in the environment in the UK is considered negligible.

**BACULOVIRUSES**

Considerations relating to the vector

47. Baculoviruses are pathogens of a range of insects and may, in certain circumstances, pose a potential threat to such species in the natural environment. In particular, the use of baculoviruses and susceptible host organisms must be given particular attention to ensure release to the environment does not occur.

48. The most commonly used Baculovirus vector utilises the highly expressed and regulated Autographa californica nuclear polyhedrosis virus (AcNPV) polyhedrin promoter modified for the insertion of foreign genes. One of the major advantages of this invertebrate virus vector is the very abundant expression of recombinant proteins in cell cultures such as Sf9 from *Spodoptera*.
Note that *S. frugiperda* is classified as a plant pest and MAFF/SERAD advice should be sought on the requirements pertaining to its use. A licence may be required for work with the insect, however, no licence is required to work with cell lines derived from it.

**Disabled or attenuated baculovirus vector systems**

49. Although the original virus was pathogenic for certain lepidoptera, the expression system is based on a deletion of the polyhedrin gene which renders the virus sensitive to insect larval gut conditions and to environmental factors. Polyhedrin negative baculovirus are also susceptible to desiccation and UV light; the survival time is in the order of minutes or hours (compared to days or weeks for wild-type, occluded, AcNPV baculovirus with normal polyhedrin genes). In addition, although the virus can infect insects if injected directly, its infectivity is far lower than the parental virus.

50. Baculovirus expression systems should not automatically be assigned to ACGM level 1 containment but the risk assessment should take the above into consideration before determining the appropriate containment. It has generally been accepted that baculoviruses are not capable of infecting vertebrate or plant cells and as such do not pose any inherent hazard to workers. Recently, however, there have been studies carried out which show that baculoviruses can express foreign genes under the control of mammalian specific promoters in human or rat hepatocytes. However the high level of expression of recombinant proteins possible with such vectors may cause workers to be exposed to pharmacologically or physiologically active products. The potential for such exposure must be examined in the COSHH risk assessment for each place of work.

**HERPES SIMPLEX VIRUS**

**Considerations relating to the vector**

51. Herpes simplex (HSV) is a double-stranded DNA virus which occurs in two closely related serotypes, HSV-1 and HSV-2, both of which are classified Hazard Group 2 biological agents. The majority of adults are seropositive for HSV. Infection is by contact, and transmission by the aerosol route is not thought to occur. Accidental infection in the laboratory is by splash to a mucosal surface, or by entry through broken skin. Except in neonates and the immuno-compromised, HSV infection is not systemic but is limited to epithelial cells at the infection site and to the sensory ganglia that innervate that site. Very rarely (< 1 in 10^6 people/year) the virus enters the CNS and causes encephalitis. Primary infection normally results in productive infection in epithelium which probably involves millions of cells. The virus travels to the sensory ganglion where further productive infections occurs, involving perhaps hundreds to thousands of neurones. Some neurones, hundreds, become latently infected. Reactivation and recurrence of latent infection requires infection by a few latent neurones and seeding of the epithelium where millions of cells may again be productively infected.

52. Latent infection, once established, is life-long and cannot be cured. If a promoter was active during latency, lifelong expression of an inserted sequence would occur from perhaps 100-1000 cells. The precise nature of long-term gene expression during latency is uncertain. Certain constructs that incorporate the virus-specific LAT (latency associated transcript) promoter are claimed to give long-term expression in sensory neurones, but heterologous promoters that are known to function in neurones appear to be silenced in the context of the latent genome. It is apparent that long-term gene expression in latency is difficult to achieve, but where long term expression is sought, this should be a factor in assessing risk.

**Disabled HSV vectors**

53. Approximately half of the 70-odd genes of HSV are dispensable for growth in vitro. Deletion or disruption of these genes has, in many cases, been demonstrated to result in substantial attenuation in the mouse. However, careful review of the evidence supporting the attenuation will be needed if particular insertion sites are used. Insertion into, or disruption of, the following genes have been shown to cause substantial attenuation:

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The gene RL1 (encoding gamma 34.5) and IE1 (encoding IE-110) lie in the inverted repeat (diploid) sequence flanking the long unique region of the genome. Deletion results in substantial attenuation (many logs growth reduction in vivo). However, recombination with a wild virus might result in a heterozygote with one normal and one deleted copy. Where an attenuating (or disabling) site and a foreign gene insert are introduced into a diploid site, the risk assessment should consider the consequences of generating a competent heterozygote by recombination with a wild-type virus.

### Replication Defective Vectors

55. Deletion mutants lacking essential replication genes (see above) have been constructed e.g. the gH vector DISC HSV-1, together with helper cell lines providing the relevant functions in trans. Mutants of this type have the potential to act as replication defective vectors and, provided the foreign gene is inserted at the site of disablement, can be considered to provide a high margin of safety.

56. No gene has yet been identified whose function is absolutely required for the establishment and maintenance of latency. All disabled or replication-defective mutants are capable of establishing latent infection if administered at a sufficiently high dose accompanied by tissue trauma. Accidental infection would result in transient expression of a vectored gene, and in most cases, death of infected cells. However, the possibility of latency after accidental infection should be considered as part of the risk assessment.

57. If it is intended to use disabled or attenuated HSV vectors, particularly when the inserted gene encodes a protein which could potentially cause harm, the risk assessment should consider:

- the rate at which replication competent virus is generated;
- whether, following high dose peripheral infection, replication competent virus could be isolated from the infection site or competent or defective virus be isolated from sensory ganglia;
- the predicted latent infection rate (as measured by in situ hybridisation) and whether competent or defective virus could be isolated by reactivation.

### Amplicons
58. ‘Amplicons’ are plasmids carrying an HSV origin and packaging site, and can be used as packageable vectors to deliver foreign genes. Since they carry no viral genes they are not cytotoxic and can be used as gene delivery vehicles. By definition they are helper virus dependent and stocks usually contain at least 50% helper virus. Risk assessment should consider the potential of the helper virus to complement the amplicon in vivo and the possibility of recombination between the amplicon and the helper. In general, the use of a disabled or attenuated helper is advisable.

**Effects of the inserted sequences**

59. Some approximate predictions can be made about the likely production of a gene product expressed from an efficient promoter. A primary or recurring infection of the epidermis would give transient delivery of a few micrograms; in the sensory ganglion this would be a few nanograms during primary infection and 10's of picograms during recurrence.

60. The nervous system is poorly understood and the view that nanograms or picograms of a particular protein ‘would have no biological effect’ either on the behaviour of the expressing cells or on the physiology of local cells is difficult to state with certainty. One exception might be ‘innocuous genes’ inserted at any site which disrupts a gene or transcript. Such genes would include reporter genes, like lacZ and CAT whose expression in all tissues in transgenic animals throughout life is known to have no pathological effect.

61. As with all viral vector systems, additional biological containment can be achieved if the cloned gene is inserted at the attenuating or disabling site. This is particularly appropriate for inserted genes with a potentially harmful phenotype.

**Effects of the inserted sequences on the characteristics of HSV**

**Tissue tropism and host range**

62. The restriction of HSV to the epithelium and sensory nerves is not due to receptor specificity since HSV is capable of binding to, and entering, a wide range of cell types. Innate defence mechanisms are thought to be of great importance in limiting the virus while specific immunity is of importance in clearing primary and recurrent infection from the epithelium.

**Altered infectivity or pathogenicity**

63. HSV very rarely causes an overwhelming encephalitis in apparently normal people, and the reason is unknown. The insertion of genes, into HSV, whose products are predicted to modify neuronal physiology should be contemplated with caution. The tendency for HSV to establish latent, rather than productive, infection in neurones may be due to the failure of immediate-early gene transcription in this cell type. Modifications designed to change the transcription factor requirements of the virus should be carefully risk assessed.

**Recombination or complementation.**

64. Recombination could occur following an accidental infection if the recipient was actively infected at the time. If the inserted gene is inserted at the attenuating or disabling site, recombination will not result in a fully competent vector. The question of whether a pre-existing infection in an individual could provide ‘helper function’ to a defective or attenuated vector is more difficult to assess. It is conceivable that accidental infection with an attenuated, disabled or amplicon vector could result in complementation by a wild virus actively replicating in the victim, and it should be noted that asymptomatic virus shedding is much more common than recurrent cold sores. Recombination between two attenuated viruses, to generate a wild type virus, is readily demonstrable in vivo by simultaneous inoculation at a single site. Complementation of a replication defective virus by a wild-type helper is difficult to achieve in vivo and has been demonstrated (with low efficiency) only by simultaneous inoculation of high doses.

**Availability of therapy or prophylaxis.**

65. Some consideration will be needed for insertion into the commonly used TK locus because thymidine kinase negative viruses are resistant to acyclovir and this therapy would be denied in the event of accidental infection. However, such viruses are still treatable using ‘Foscarnet’ in the unlikely event that infection with TK- HSV should need treatment.

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66. Humans are the only natural host for HSV. Many other species can be infected experimentally, but these appear to be "dead end" hosts. The virus is fragile, and is rapidly inactivated by desiccation, lipid solvents and mild detergents. The fact that contact is required for transmission attests to the instability of the virus outside the host.

POXVIRUSES

Considerations relating to the vector

67. Vaccinia virus recombinants are useful tools for the molecular biologist and immunologist. High levels of expression can be achieved facilitating biochemical, biological and immunological characterisation of foreign genes. Several strains of vaccinia have been derived from the original vaccine material as part of the smallpox eradication campaign, common examples include Wyeth (also known as the New York Board of Health strain, NYBH), Lister and Copenhagen. Primary vaccination in humans causes a vesicular lesion at the site of inoculation usually associated with a general infection and, rarely, a viraemia between the third and tenth day. After about 7 days, the lesion crusts over and detaches, leaving a characteristic scar. Despite millions of individuals being vaccinated without effect even vaccine strains such as Lister and Wyeth can cause infections in humans. Figures from the USA in the 1960's show that out of 14 million vaccinations there were 572 hospitalisations, 9 deaths and many less severe complications. The rate of severe adverse reactions is approximately 1 in 50,000 vaccinations.

68. Vaccinia is categorised as a Hazard Group 2 biological agent in recognition that it may cause particularly severe disease in people with active skin disorders such as eczema or psoriasis or in immuno-compromised individuals such as those infected with HIV.

69. The complex and large genome of vaccinia (over 175Kb depending on the strain) contains an estimated 150-200 genes many of which are necessary to enable the virus to replicate in the cytoplasm of infected cells. Infectious progeny virus can be detected approximately 6 hours after infection and continue for about 48 hours. With the commonly used strains, the progeny virus are released by eventual cell lysis. For laboratory workers, ingestion, parenteral inoculation, and droplet or aerosol exposure of mucous membranes or broken skin are possible routes of infection. Laboratories working with vaccinia and other poxviruses should have suitable local rules to control these potential sources of infection.

Disabled or attenuated vaccinia virus

70. Defective vaccinia strains have been produced to reduce the incidence of complications in vaccination campaigns. For example, strain LC17m8, derived from the Lister vaccine strain, is temperature sensitive and shows lower neurovirulence. Strain MVA (Modified Virus Ankara) is multiply attenuated, containing six major deletions totalling 31Kb and will not replicate in human cells. NYVAC (New York Vaccinia, derived from Copenhagen strain) is deleted for TK, haemagglutinin (HA) and over 14 other genes. Strains MVA and NYVAC are unable to produce infectious virus in humans and may be considered for use at ACGM level 1 depending on the nature of the insert.

71. The use of the thymidine kinase (TK) gene as an insertion site, creating a Tk-minus phenotype, is believed to reduce the virulence of the virus in mice but it is debatable whether this should be taken to imply lower virulence in man, or whether this should allow a down-grading of categorisation. Deletion of the 19kDa epidermal growth factor (EGF) homologue gene results in marked reduction in pathogenicity, although the viruses grow well in tissue culture.

Avipoxviruses

72. Fowlpox, pigeonpox and canarypox have been used as vectors for foreign genes generally with the intention to use them as vaccines. Avipoxviruses are restricted to growth in avian species. Whilst high multiplicity of infection of mammalian cells with fowlpox virus (FPV) causes cytopathic effect, there is no evidence or productive replication of the virus in those cells. Consideration of spread in the animal population should be taken into account in a risk assessment but in general for innocuous foreign gene inserts recombinants can be handled at the same category as the parent virus.

73. Attenuated derivatives of FPV (TROVAC) and canarypox virus (ALVAC) have been demonstrated
to be non-virulent in a variety of immuno-suppressed animals and human volunteers. Depending on the nature of the inserted sequences, fowlpox- and canarypox-based recombinant viruses can be handled at ACGM containment level 1. Although the replication of these viruses is abortive in mammalian cells, there is clear evidence that the viruses can infect mammalian cells, albeit at relatively low levels. Due consideration must therefore be given to expression of toxic gene products from inserted foreign genes.

74. There is a long, safe history of vaccination of chickens with attenuated strains of avipoxviruses. Such attenuated strains would be considered to be non-pathogenic. Wild-type strains, are pathogenic for birds. Attenuated vaccine strains are available from various commercial suppliers. The most extensively studied and readily-available FPVs are attenuated derivatives of virulent strain HP-1, isolated by Mayr & Malicki in Munich (1966). Viruses passaged more than 200 times (i.e. HP1-200, HP1-220) in chick embryo fibroblasts (CEFs) are considered to be attenuated and are the basis of some commercial vaccines. They still replicate well in chick embryos and replicate moderately well in CEFs. They posses residual pathogenicity for chickens, however, resulting in systemic lesions after intravenous inoculation of lung lesions after aerosol infection. Virus passaged in CEFs more than 400 times (i.e. HP1-400, HP1-438, HP1-440) are considered to be apathogenic by all routes. These viruses replicate well in CEFs (or in the permanently, transformed quail cell line, QT-35) but poorly in chick embryos.

75. Canarypox virus has been less well studied but similar, extensively-passaged, attenuated strains have been derived by Mayr (e.g. KP1-558).

76. Other poxviruses have been used as vectors e.g. suipox and sheeppox/goatpox. Work with these viruses is regulated by MAFF (and equivalent devolved administrations) (see Annex II) and should not be undertaken except in the appropriate facilities.

77. Particular attention must be given during the risk assessment to the insertion of genes that code for proteins that may have adverse physiological or pharmacological effects in vivo. In all known cases, poxviral infection kills the infected cell and expression of toxic products will only be an issue if they may affect other cells when released from the cell (either biosynthetically or upon death of the cell).

78. Of particular importance are recombinants intended to investigate autoimmune responses or allergenicity in animal models. In such cases, the possibility of autoimmune or allergenic reaction in persons handling the virus should be carefully assessed and consideration should be given to the use of non-replicating poxvirus vectors.

79. Lymphokine genes have been inserted into vaccinia with a view to improving the immune response and decreasing complication rates. These include IL-1, IL-2, IL-6 and g-interferon. In some cases, such recombinants were highly attenuated with respect to the parental virus. The risk assessment should, however, consider carefully the likely effect of any other inserted proteins in determining the appropriate containment and control measures.

80. When considering the use of oncogenic inserts, as the poxviral infection is usually lethal to the cell (even to non-permissive cells) transformation of the cell is unlikely to occur.

Effects of the inserted sequences on the characteristics of poxviruses

Tissue tropism and host range

81. Within the poxviruses, host range varies in nature and extent. Vaccinia host range includes humans and animal species such as cattle, cats, rodents, rabbits and pigs, although the virus does not appear to occur naturally in humans and has no animal reservoir. At least three host range genes can be identified in poxviruses; vaccinia contains C7L and K1L, the insertion of a third, CHO hr gene, allows the virus to grow on Chinese hamster ovary (CHO) cells. Deletion of the C7L gene and presence of CHO hr allows growth on rabbit kidney cells. None of the host range genes are receptor attachment proteins.

Altered infectivity or pathogenicity

82. Many poxvirus genes are dispensable for growth in vitro. Deletion or disruption of these genes has, in many cases, been demonstrated to
result in substantial attenuation in the mouse. However, careful review of the evidence supporting the attenuation will be needed if particular insertion sites are used. Insertion into, or disruption of, the following genes have been shown to cause attenuation:

- Thymidine kinase (gene J2R)
- haemagglutinin (gene A56R)
- Formation of extracellular virus (EEV) (gene B5R), 14K fusion (A27L), envelope antigen (F13L)
- Virus growth factor
- Complement C4b binding protein
- Complement control (C3L)
- DNA ligase (A50R)
- Ribonuclease reductase (N1L)
- Steroid dehydrogenase (A44L)
- A-type inclusion body protein ATI (A26L)

83. In one unusual case it has been shown that the deletion of the B15R gene actually exacerbates some pathogenic properties as compared to the wild-type virus. Deletion of this gene results in an earlier onset of illness with more severe symptoms.

Recombination or complementation

84. Recombination between modified poxvirus and other poxviruses is dependent on DNA replication, co-localization of replication and DNA homology between the two viruses. With vaccinia, the likelihood of recombination in vivo is extremely low because the only natural infection with related orthopoxvirus would be monkeypox which is largely restricted to Zaire. Recombination with other poxviruses, such as orf or molluscum contagiosum has not been observed in vitro.

Availability of therapy or prophylaxis

85. Those working with vaccinia virus should be familiar with the ACGM/ACDP guidance on vaccination\(^5\) issued in 1990. This recommends that smallpox vaccine should not be given to those who work with vaccinia virus or related poxviruses except:

- for work with monkeypoxvirus,
- in the light of a case-by-case risk assessment. (Examples include work with modified infectious human poxvirus where there is a significantly increased hazard due to enhancement of infectivity/pathogenicity or the presence of an expressed insert; large-scale work with infectious human poxviruses, inoculation and work with animals.); and
- where the person requests it.

Further details can be found in the joint ACGM/ACDP publication.

Risk assessment for environmental protection.

86. Vaccinia virus (and other poxviruses) have the capacity to survive for considerable periods in dried material such as detached vaccination scabs. Vaccinia virus may replicate in a number of mammalian species and there are documented cases of transmission from recently vaccinated humans to wild and domesticated species. Recent experience with large scale field trials of an attenuated vaccinia-rabies vaccine in wild foxes have shown little evidence of spread to other species and the experience of the smallpox eradication campaign indicated that vaccinia is unlikely to become established in the wild. Nevertheless, the risk assessment should consider the possible effects on other species which could be infected following an accidental release into the environment. In particular, close attention should be paid to the disposal of infected waste material and the containment measures for any animals which may be infected with vaccinia viruses.

87. The natural history of cowpox virus has yet to be fully elucidated but it is clear that the virus has a much wider host range than vaccinia and this should be taken into account in any risk assessment. Small wild rodents are known to be carriers of poxviruses and can be carriers of cowpox virus. This may be the source of cowpox virus isolated from domestic cats.

88. The avipox host range is limited to avian species; there has only been one report of an


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avipoxvirus isolated from a mammal. The virus was an atypical avipoxvirus and was isolated from an already seriously ill rhinoceros in a zoo. Avipoxviruses have been isolated from a number of avian species (e.g. fowlpox, canarypox, pigeonpox, juncopox, quailpox, mynahpox). Some can infect species other than their normal hosts. There are no clear indications of pathogenic consequences (indeed pigeonpox is naturally attenuated in chickens and has been used as a vaccine) but use of attenuated avipoxvirus strains would be recommended to reduce the risk of environmental spread to other avian species.