Work with naked DNA or RNA (including oligonucleotides, siRNA, miRNA, sequences that code for highly biologically active molecules and full length viral genomes)

Guidance

1. Introduction

The following guidance refers to the handling of naked DNA or RNA. This may be as part of the process of genetic modification or where the nucleic acids are used for in vitro studies including other techniques such as vaccination with naked DNA and in vitro and in vivo delivery of oligonucleotides or siRNA.

Work with naked DNA or RNA and oligonucleotides is widely regarded as being safe. However, it is generally agreed that there is an increased risk when handling DNA or RNA that codes for highly biologically active molecules, full length viral genomes (cloned or isolated directly from a virus) and active biological modifiers such as siRNA. Even with these types of naked DNA or RNA the risks can be over-estimated as there needs to be an effective means by which the DNA or RNA can gain entry into a cell and cause harm. For RNA in particular there are further factors that complicate risk assessment, the primary issue being the likely stability of the RNA considering the ubiquitous nature of RNAse in the environment.

2. Definitions

2.1 Highly Biologically Active Molecules

The following types of molecules (not intended as a comprehensive list) are often considered as highly biologically active (see the WHO Laboratory biosafety manual, third edition, 2004). This would also include genes in the signal transduction pathways of these molecules, specifically designed regulators of the genes such as siRNA and possibly molecules that interact with these pathways such as miRNA's.

1. Toxins
2. Immune modulators including cytokines
3. Hormones
4. Gene expression regulators
5. Oncogenes
6. Allergens

2.2 Oncogenes

In order to illustrate the issues involved this section will discuss oncogenes which have been the focus of concerns for a number of years. However it should be remembered that each gene has a different biological activity and there

is likely to be a level of uncertainty associated with any assessment.

Oncogenes can be considered as sequences whose gene products induce cells to become tumour cells. While a more precise definition is difficult because of the many different features of transformed cells, some of the following classes of genes can be considered to be oncogenes:

2.2.1 Viral oncogenes and their cellular homologues e.g. Ras, Myc, Src, Sis etc (note: viral oncogenes, such as SV40 T antigen, adenovirus E1A and E1B, are not considered oncogenic when expressed in their natural viral setting).

2.2.2 DNA sequences that generate tumors in animal experiments,

2.2.3 DNA sequences that transform mammalian cells *in vitro*, viz

- Immortalization sequences
- Growth-regulating sequences
- Sequences that result in the loss of contact inhibition or cause cells to become tumorigenic in animal experiments.

There will always be some debate about many of these genes and whether they are "fully oncogenic" For example those genes involved in steps along a pathway to a cancerous cell as well as genes that act in concert. EBV transforms B-cell very efficiently but the risks associated with handling individual EBV genes involved in the immortalization process such as LMP1 and EBNA2 is difficult to assess because individually they are less effective than when part of the viral genome.

There is no direct evidence that oncogenic DNA or RNA molecules can lead to tumours in humans although some evidence exists in animals (Burns *et al*., Oncogene 1991; 6 (11): 1973-1978: "Transformation of mouse skin endothelial cells in vivo by direct application of plasmid DNA encoding the human T24 H-ras oncogene"). The experimental protocol in this paper involved extreme conditions that are unlikely to be repeated in a laboratory setting where large doses of H-ras were applied to scarified skin. However the development of cancer is a multi-stage process that requires the activation of oncogenes and the inactivation of tumor-suppressor genes and an oncogene that is introduced into a cell in a stable manner can be considered as bringing the cell and its descendants a step closer to the formation of a tumor. The effectiveness of DNA vaccines (injected as naked DNA) as demonstrated in animal models and clinical trials does imply that handling naked oncogenic DNA should therefore be considered as potentially hazardous.

3. Bacterial and Viral Nucleic Acids

There are a number of important aspects to consider when nucleic acid is extracted from pathogenic micro-organisms.

Firstly, it is important to consider whether the preparation is likely still to contain any of the intact parent micro-organisms. If nucleic acid is extracted from a culture of a hazard group 3 pathogen then the material should not be removed from Biosafety Level 3 (CL3) until a stage in the extraction process when all the

parent organisms are known to have been destroyed. Alternatively the culture can be killed first and the extraction can be done elsewhere, however in this case a rigorous validation of the kill method would be required with stringent controls to ensure effectiveness of the procedure on every occasion it is used.

Secondly, an assessment should be made of whether the naked DNA or RNA is infectious once extracted. This is not a concern with bacterial DNA but may be in the case of some full length viral genomes

Naked viral DNA can be infectious but it is much less so than the intact DNA virus equivalent. Different DNA viruses will have different levels of infectivity as naked DNA, but it would need several orders of magnitude of naked DNA molecules to get one infection to take place in a host cell (assuming it is not being helped by artificial means). This is because the intact virus particles have all the machinery to attach to and penetrate the cell whereas naked DNA has none of this machinery. Also, many DNA viruses carry important proteins or enzymes in their structure that are required to be present when the intact virion gains entry to the host cell.

Positive-stranded RNA viruses are usually described as containing infectious RNA. RNA extracted from flaviviruses and alphaviruses for example, are infectious when inoculated intracerebrally into newborn mice. There is the argument that this means this RNA should be handled at Containment Level 3 in the same way as the normal virus from which it was derived. However this is not proportionate to the actual risk. Naked RNA is extremely labile. It takes only the minutest quantity of RNAse enzyme, which is virtually everywhere in the environment, to destroy the intact molecule. Furthermore, it has been estimated that the number of molecules of RNA that are equivalent to a single infectious unit is approximately 1×10^{10} (i.e. approx. 10,000,000,000 RNA molecules are required to initiate an infection that an intact single virus particle can initiate).

If the nucleic acid contains an intact full length viral genome, that might be infectious as naked nucleic acid, for example, if it were to be injected in a sharps injury, then a precautionary approach should be taken. However, it is probably not necessary to apply the same level of containment as the parent virus. Control measures should be directed at providing extra protection against percutaneous inoculation, and contamination of the skin, mucous membranes and laboratory working surfaces.

3.1 Cloned Full Length Viral Genomes

Techniques have been developed to clone even large viral genomes and rescue infectious virus. For example adenoviruses (30-38 Kb) have been cloned in yeast, in baculoviruses or as bacterial artificial chromosomes (BACS) in *E.coli* and can be rescued at high efficiency from the cloned naked DNAs. Many herpesviruses which range from ~150Kb (HSV-1) to 230Kb (CMV) can also be cloned in BACS as full length genomes. Not only can disabled full length clones be used as vaccines to protect against the parent virus but transfection/ electroporation of microgram quantities of the naked wild type viral BAC DNA into cells yields the corresponding virus.

RNA synthesized *in vitro* from full length genomes can be infectious e.g. polio, but as indicated previously the stability of the RNA and the number of molecules required to initiate

infection should be taken into account in any assessment of risk.

4. Risk Assessment

In undertaking a risk assessment of specific sequences it is important to consider what is likely to happen if the naked DNA were to gain access to the body and if having gained access a gene is likely to be expressed efficiently. Sequences with strong eukaryotic promoters attached are therefore likely to be considered more of a risk that the same sequence lacking a promoter. As a first step the hazard should be identified and a worst-case scenario of the possible consequences of integration or expression of the DNA should be given. This should then be countered by due consideration of the likelihood that integration or expression would occur and whether any harm would result. The risk should take account of both the potential hazard and the likelihood of harm occurring in the event of exposure. It should also be made clear in the risk assessment whether the work involves a known, full length, functional highly biologically active gene or a fragment or altered form of such.

A comparison can be made with risk assessments for genetic modification (GM) work because risks vary and can increase significantly when highly biologically active gene sequences are used in some types of GM work. This variation in risk is due to changes in the likelihood of harm resulting because of the specific activity. The cloning of an oncogene in disabled *E.coli* would generally be regarded as low risk warranting Containment Level 1 precautions as the potential to cause harm is usually minimal. However, packaging of the same oncogene in a delivery system such as a viral vector with a host range capable of infecting humans, particularly if the oncogene is linked to strong promoters or enhancer sequences that function in mammalian cells, would present a significantly higher risk and in some circumstances may well require Containment Level 3 precautions.

Other forms of nucleic acid such as *in vitro* produced RNA, oligonucleotides, synthetic siRNA, miRNA and ribozymes can be considered in terms of their properties i.e. their stability, susceptibility to RNAse, whether produced *in vivo* or *in vitro*, target gene activity, likely level of modulation of the target gene etc

5. Control Measures

The risks associated with handling oligonucleotides, naked DNA and RNA are generally regarded as low since they are unlikely to traverse the natural defence barriers of the body. In addition naked RNA is extremely labile and rapidly destroyed in the environment. The only occasion naked DNA/RNA is likely to gain access to the body is when the skin is punctured, as in the case of a sharps injury, and the material is effectively injected in to the body. Therefore, of prime importance in the control of exposure is the avoidance of sharps in the manipulative procedures. Particular care must also be taken if the naked DNA/RNA is used in solvents which have the ability to penetrate the skin (this should be detailed in the risk assessment and appropriate gloves selected) or are in membrane fusing agents etc.

If the DNA/RNA is likely to contain harmful sequences then consideration should be given to whether it may be possible to include a stage in the protocol whereby the DNA/RNA is denatured. An example of this type of procedure would be heat treatments in excess of 90°C. A denaturing stage will eliminate any potential for

expression and therefore, the hazard will be minimal. Denaturing techniques should not be confused with protein denaturing steps such as phenol chloroform treatment which do not affect the nucleic acid.

In many countries, in legal terms, naked DNA/RNA itself is not regarded as a biological agent. They are viewed as complex chemicals and therefore there are no minimum standards to apply in the form of specified containment levels that would be legally binding if they were biological agents. Therefore, in the case of potentially harmful DNA or RNA, control measures should be assigned commensurate with the risk. The precautions given in Appendix 1 are recommended as a minimum for work with highly biologically active molecules and related DNA sequences and full length viral genomes that may be infectious. These measures correlate broadly with basic procedures for work in laboratories at Biosafety level 2 (Class 2).

Appendix 1

Particular attention should be given to the following precautions for work with DNA sequences encoding highly biologically active molecules and full length viral genomes that may be infectious:

- Access to the laboratory where potentially harmful DNA/RNA is handled should be limited to authorised personnel and designated workers.

- Persons undertaking work with potentially harmful DNA/RNA must receive basic information and training prior to starting work. They should be trained in good laboratory techniques before commencing work and should be fully aware of the potential hazards of such work.

- Laboratory coats must be worn at all times in the laboratory and be removed when leaving. These should be kept apart from uncontaminated clothing.

- Bench surfaces should be impervious to water and easy to clean.

- A wash basin must be provided and preferably sited near the exit of the laboratory. The taps should be of a type which can be operated without being touched by hand. Hands must be washed before leaving the laboratory.

- Eating, chewing, drinking, smoking, etc should be forbidden in the laboratory and mouth pipetting must not take place.

- Potentially harmful DNA/RNA sequences should be handled at designated benches within the laboratory. These should be kept clear of any unnecessary equipment. On completion of the work there should be a rigorous clear up procedures in place to ensure the area is left safe. All work surfaces must be cleaned after use and waste materials removed.

- Gloves must be worn for all work with potentially harmful DNA/RNA sequences. Gloves should be chosen also taking into account their resistance to any chemicals in use. They should be changed regularly and special attention paid to the danger of glove puncture. Gloves worn for this work should not be worn elsewhere. The use of gloves should not preclude the covering of cuts by suitable dressings.

- Sharps must not be used for potentially harmful DNA/RNA work, except where essential, such as for animal inoculation. Glassware must not be used where plastic alternatives exist.

- All experimental procedures involving potentially harmful DNA/RNA should be performed so as to minimise aerosol production. Procedures which are likely to generate aerosols such as the use of blenders, sonicators, vigorous shaking and mixing etc. must be conducted under effective engineering controls including suitable local exhaust ventilation systems (e.g. fume cupboard or ducted vent over the equipment) if appropriate, or in equipment which is designed to contain the aerosol. The suitability of such systems should be determined as part of the risk assessment. However, the control measures utilised for such work must not accentuate the risk in other workplaces or in the outside environment. Where there may be an additional microbiological hazard, a microbiological safety cabinet must be used.

- Potentially harmful DNA/RNA should be securely stored in marked refrigerators, cupboards or rooms when not in use.

- Arrangements should be made for immediate surface decontamination after spillages. Dilution of spillages with detergent and careful disposal of solid waste by incineration is recommended.

- Wherever possible, potentially harmful DNA/RNA should be denatured or destroyed, for example by breaking into biologically inactive fragments or heat treatment, prior to disposal.

- All accidents or incidents should be reported to and recorded by the person responsible for the work. The University Health and Safety Department and the Occupational Health Unit should be informed immediately in the event of any accident where exposure to potentially harmful material may have occurred. A full accident record should be prepared and forwarded to the Health and Safety Department as soon as possible.

Note: - whilst it may not be appropriate to assign naked nucleic acids to a specified containment level (since these are not biological agents) the above precautions are comparable to Biological Safety Level 2 standards