Non-technical summary: Virus host interaction studies for control of avian tumour diseases

Project duration
5 years 0 months

Project purpose
(a) Basic research
(b) Translational or applied research with one of the following aims:
(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
(iii) Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
(c) Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
(d) Protection of the natural environment in the interests of the health or welfare of man or animals

Key words
Marek's disease virus, Avian Leukosis Virus, Host-virus interaction, Recombinant vaccine, Immune response

Animal types
Domestic fowl (Gallus gallus domesticus)

Life stages
Embryo, neonate, adult

Retrospective assessment
The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits
Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing. What's the aim of this project?
The broad purpose of this project license application is to gain better understanding of the mechanisms of diseases characterized predominantly by cancer of different cell types, caused by a group of pathogens, commonly referred to as cancer-causing or oncogenic viruses, which include Marek’s disease virus, avian leukosis virus and reticuloendotheliosis virus.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished. Why is it important to undertake this work?
These cancers are complex diseases involving multiple steps and factors, and there are many unknown causes and steps through which cancer develops. Because of the complexity and multisystem involvement of these diseases, there are no in vitro models. Hence these studies can only be conducted in experimentally-infected birds and are very important to develop better intervention strategies to control and prevent cancer in chickens.

What outputs do you think you will see at the end of this project?

This application is part of the overall research aimed at understanding the molecular mechanisms of cancer induced by these important group of viruses, particularly on supporting our laboratory based research on specific virus-host interactions associated with pathogenicity. The major output from this project will be advancement of scientific knowledge disseminated through scientific publications, research output in the form of new diagnostics and intervention strategies including new vaccines and eradication procedures for more effective control.

Who or what will benefit from these outputs, and how?
Avian oncogenic viruses are associated with huge economic losses and major welfare problems in the poultry industry. Diseases such as Marek's disease (MD) have a worldwide distribution and are reported to cause economic losses of US$ 2.0 billion annually. MD vaccination today is almost a routine practice of the poultry industry in most parts of the world, without which it is impossible to maintain healthy poultry production. Although vaccines are still valuable in preventing losses from MD, increasing virulence of Marek's disease virus (MDV) isolates remains a major threat. We demonstrated that herpesvirus of turkey's (HVT) vaccines helped the spread of more virulent strains thereby providing opportunities for evolution of virulence (2). Our recent success with the CRISPR/Cas9-based gene editing of the MDV-transformed cell lines has given immense opportunities to investigate the role of virus-host interactions in situ in these cell lines and eventually in vivo. In addition, gene editing approaches that we have developed could help innovations in multivalent vaccine development to offer simultaneous protection to multiple avian diseases. Some of our current collaborative projects with the leading poultry vaccine industry could also help in faster translation of our research findings to the field. Similarly, recombinant viral vector-based immunoprophylaxis will be a novel approach against major avian diseases.

Similarly, other tumour diseases such as avian leukosis and reticuloendotheliosis also remain major threats to the poultry production in many countries. The sudden emergence and spread of the new subgroup J associated with myeloid leukosis, and the continuing re-emergence of antigenic variants associated with syndromes such as haemangioma in both broiler and layer flocks are examples of great concern. Hence continuing research is essential to understand the molecular virus-host interactions of these viruses, as it will help to maintain expertise and develop novel control strategies. In addition, our recent success with in vitro induction of genetic resistance in DF-1 cells (12, 13), an innovative approach for de novo generation of chickens with induced genetic resistance to different ALV subgroups. The new Licence will give us the opportunity to test such birds for genetic resistance to infection.

Summary of benefits
1. In the short-term, the licence will give the opportunity to understand the complex virus-host interactions involved in these virus-induced cancers. Advances in molecular tools for global analysis of gene expression will allow us to gain significant insights into the molecular cancer pathways, pathogenic determinants and mechanisms of diseases caused by oncogenic viruses. This will benefit researchers from biosciences by increasing understanding of tumour pathogenesis and developing innovative approaches in disease control.

2. In the medium-term, the licence is essential to the development of next generation MD vaccines to curb the continuing increase in virulence and emergence of hypervirulent pathotypes, which are threatening the sustainability of the control strategies. Similarly, development of novel multivalent recombinant vaccines that can simultaneously protect

against a number of avian viral pathogens will be a very valuable benefit to a number of stakeholders of the poultry industry as well as the vaccine manufacturers. Improved control of diseases by avian oncogenic viruses will help improving animal welfare and important for global food security.

3. In the long-term, research towards developing de novo genetic resistance to diseases such as avian leukosis, where vaccine-based control methods do not exist, will have immense benefit to the industry in the fight against some of these devastating diseases, particularly in ODA countries where the economic losses from such diseases are very high.

How will you look to maximise the outputs of this work?
Our research group has a long standing close interaction with different stakeholders of the poultry industry including breeding companies and vaccine manufacturers. For example, we work with major poultry breeding companies and have helped in controlling these group of diseases. We also work closely with all the major poultry vaccine manufacturers. This would allow us to translate the research findings for commercial applications. Our group is also the WOAH Reference Laboratory (MDVRL) for Marek's disease and has interaction with animal health regulatory bodies in many countries. We also are leading major international research networks including Global Alliance for Research on Avian Diseases (GARAD) and UK-China Centre of Excellence for Research on Avian diseases (CERAD). All these networks and research collaborations will help to maximize the output of this work.

Species and numbers of animals expected to be used
Domestic fowl (Gallus gallus domesticus): 4600

Predicted harms
Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.
Chickens are the natural hosts of avian oncogenic viruses. Natural infections by these viruses occurs to embryos and neonates. In order to get the detailed output of virus-host interactions and vaccine responses, it is important that we use chickens and the embryos or neonates.

Typically, what will be done to an animal used in your project?
At the end of the experiment (usually 8 weeks after infection), birds will be killed by a Schedule 1 method or alternatively, birds may have their necks dislocated, and will then be exsanguinated via decapitation (regulated procedure)

What are the expected impacts and/or adverse effects for the animals during your project?
Animals included in this project will be subjected to experimental infections by oncogenic viruses. As naturally occurring endemic diseases in many countries including the UK, clinical diseases in these experimentally-infected birds are similar to those occurring in natural infections. Although small proportions of birds may suffer from clinical disease with moderate severity, most of the animals suffer from a mild chronic disease with weight loss, reduction in appetite and tumours. With most experiments of an 8-week duration, some of the animals may not have developed any symptoms at all.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?
Maximum severity of the protocols in this application is moderate. This level of severity is reached only in birds infected with acutely transforming retroviruses or very virulent MDV pathotypes. In experiments with these viruses, most of the animals will reach moderate levels of severity. However, the robust clinical scoring methods and frequent inspections will help in majority of these birds not going to the maximum severity but humanely killed by a schedule 1 method.

Infection with less virulent MDV and non-acute retroviruses, most birds will experience mild chronic disease.

What will happen to animals at the end of this project?
Killed

Replacement
State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?
Diseases caused by these group of oncogenic viruses are exclusively seen in poultry. The oncogenic process and tumour formation are very complex events with the involvement of network of multiple genes that are associated with tumour suppressor functions, activation, signal transduction, immune checkpoint modulation etc. Because of the complex nature of oncogenesis, it is difficult to have suitable in vitro models that are comparable to in vivo disease models. Hence experiments in the natural avian hosts are essential.

Which non-animal alternatives did you consider for use in this project?
For some acutely-transforming viruses, cells from animals can be transformed in vitro and some aspects of the molecular mechanisms of induction of cancer can be studied. Similarly, for Marek's disease, some aspects of the virus-host interactions can be studied using transformed cell lines derived from lymphomas induced in the infected birds.

Why were they not suitable?
These in vitro models of transformation only gives a part of the story involved in virus-host interactions and mechanisms of transformation. As the lymphoma and other tumours induced by oncogenic viruses are complex involving multiple systems, none of the in vitro systems can reproduce the authentic multisystem involving lymphomas and other tumours induced in vivo from experimental infections. Moreover, there are no in vitro transformation models for Marek's disease virus. Studies on MDV-transformed cancer cells only gives the virus-host interactions in an already transformed cell, and not the dynamic changes in the neoplastic transformation process.

Reduction
Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?
The total number of animals requested is based on the estimated numbers used in the previous years. Experiments using the infectious agents would require comparisons to be

made with those of uninfected control birds. Quantitative data will be compared using analyses of variance, t tests and/or chi-squared analysis. Final size of the experimental groups will be determined on the basis of procedures described for statistical methods using advice from statisticians. The number of birds in each group will be determined using 'power of experiment' calculations based on q-PCR data (means & standard deviations) from previous experiments. Typically, the group sizes of animals for experimental studies will be between 6 and 10 birds, based on calculations from previous studies. For example, using q-PCR measurement of the viral genome copies per 104 cells (expecting a 5-fold difference required to be detected as the criteria), we have observed that a group size of 8 gave statistical difference at a 'p' value of 0.05 and a power of 80%.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?
Further reduction will also be achieved by using the same 'control' group for more than one experiment. Birds allocated to different groups with randomised wing-band numbers. With regard to the genetic variability of the host and the measures to control it, the proposed group sizes are considered appropriate, because the modern commercial broiler/layer birds have comparatively less heterogeneity based on the recent studies on MHC variability in birds from a number of commercial breeding companies (Kaufman, personal communication). Moreover, we will aim to use inbred lines of chickens where possible which will reduce the variability significantly. Gene-edited birds proposed to be used in a few experiments also have limited genetic variability as they are usually generated from single founder birds. Advice on experimental design and number of animals required will be sought from Statisticians at Pirbright and will also make use of the N3CR's Experimental Design Assistant (EDA) https://www.nc3rs.org.uk/experimental-design-assistant-eda.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
Experiments will be designed to keep the animal usage to the minimum, but without compromising the validity of the research findings. Most of our experiments are carried out using well characterised viruses using chicken lines with limited heterogeneity, allowing us to use minimum number of birds in different groups. Our long experience in these disease models will help to decide on the numbers needed. Moreover, we have access to specialist mathematical biologists at the Institute who advise us on the minimum numbers per group to achieve statistical significance of our data. It is a routine part of experiment planning to have approval from such experts on our animal experiments. Where control groups are required we will perform as many concurrent experiments as is practically and scientifically possible so that the same control groups can be utilised for achieving reduction in animal usage. We have not proposed to use rabbits or mice for the production of antibodies in this project (compared to the previous licence), partly from the availability of alternatives, such as the Adhiron technology. Thus, there has been an overall reduction of 10% in poultry numbers. 100% reduction in the use of rabbits and mice for antibody production.

Refinement
Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of our experiments are carried out using well characterised viruses using chicken lines with limited heterogeneity, allowing us to use minimum number of birds in different groups. Our long experience in these disease models will help to decide on the numbers needed. Moreover, we have access to specialist mathematical biologists at the Institute who advise us on the minimum numbers per group to achieve statistical significance of our data. It is a routine part of experiment planning to have approval from such experts on our animal experiments. Where control groups are required we will perform as many concurrent experiments as is practically and scientifically possible so that the same control groups can be utilised for achieving reduction in animal usage. We have not proposed to use rabbits or mice for the production of antibodies in this project (compared to the previous licence), partly from the availability of alternatives, such as the Adhiron technology. Thus, there has been an overall reduction of 10% in poultry numbers. 100% reduction in the use of rabbits and mice for antibody production.

Why can’t you use animals that are less sentient?
Chickens are the natural and susceptible species to avian oncogenic viruses. Cancer is a highly complex, multifactorial, multistep dynamic process involving several cell types and events. There are no complete in vitro models that can simulate this complexity. Hence there are no non-animal alternatives that can completely replace the use of birds. Similarly, the immune responses to these diseases can also be effectively studied only in an infected bird, again due to complex nature of the responses. However, we have tried to use alternatives wherever possible. For example, we have generated a number of cell lines from the cancer tissues derived from the infected birds. These have been used for a number of studies to examine the molecular changes that occur in the cancer cell, which are very similar to that seen in the primary cancers induced by these viruses in vivo in natural infection models. We are also using these in vitro systems for most of the recent gene editing work, to help identifying the genes that are important for inducing and maintaining the cancer cells.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?
This project benefits from the long-standing expertise of scientists who have spent many years working with oncogenic viruses and animal models. We have developed robust clinical scoring systems to accurately identify the stages of infection and appropriate humane end points. Chickens are monitored twice daily (or more frequently as required by the clinical scoring sheet) and humanely killed by a trained personnel in an ante-room so as not to distress the other chickens in the pens.

Wherever possible we will aim to carry out maximal observations of welfare of the infected birds. At our establishment, we have the expertise of a number of groups working on other avian diseases. Their expertise and experience with the clinical scoring systems will be used when the efficacy of recombinant vaccines are evaluated.

The birds used in this research will be housed either in floor pens or in isolators depending on the experiments and types of samples (such as the infected dust) to be collected. We have refined the Marek’s disease transmission experiments by changing from the isolators to the floor pens based on the data from the pilot experiments which showed comparable to those from isolator experiments.

Provision of enrichment is a priority at the Institute including for those birds housed in the isolators. Foraging, scratching and pecking are all important behaviours to chickens and so we provide our birds with substrate on the floor to allow foraging and dustbathing and toys to

enable them to express their species-specific behaviour. We also in most cases provide more space than that is legally required within the Home Office Code of Practice. Animal facilities are managed by our Animal Technicians who are experienced specialists in the care of animals. They are all trained in daily animal handling, husbandry.

Wherever possible, we have also carried out further refinement steps by using chicks derived from vaccinated parent flocks, and the maternal antibodies usually give better protection from early clinical disease.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
We have designed very accurate clinical scoring systems and humane end points for each of the animal experiments. These robust systems have refined the experiments significantly to reduce suffering and improve welfare.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?
As a member of AWERB we constantly discuss 3R. We also follow up the guidelines from NC3R. Studies in the past several years by many laboratories have confirmed that there is no alternative to in vivo animal models to study virus-host interactions in the pathogenesis of and immune responses to oncogenic viruses. Retroviruses are RNA viruses that reverse transcribe into DNA provirus which integrates into the host genome. Most of the pathogenic (oncogenic) effects of retroviruses are induced by the proviral DNA form that causes the induction of host oncogenes adjacent to their integration sites. Furthermore, the oncogenic process and tumour formation are very complex events with the involvement of network of multiple genes that are associated with tumour suppressor functions, activation, signal transduction, immune checkpoint modulation etc. Because of the complex nature of oncogenesis, it is difficult to have suitable in vitro models that are comparable to in vivo disease models. Because of this, it is imperative that we will have to conduct animal experiments for our research on these important pathogens. Marek's disease virus (MDV) also has a unique tropism for lymphoid cells for the induction of tumours. MDV can be grown in cultured chicken fibroblast cells in cell culture dishes and we use this system to grow up large stocks of the viruses and to make mutations in the viruses, without needing to use chickens. However, in the chicken, the natural target cell for the virus is the lymphocyte and these lymphocytes do not grow well in culture (unless they are derived from MD tumours), so we cannot study either the natural primary infection or the formation of tumours in vitro. Similarly, the different facets of MDV infection dynamics involving multiple cell types with distinct interactions and outcomes also cannot be studied in any in vitro models. Examination of the protective effects of vaccination has to be also performed in chickens in the absence of other in vitro models.

Although there are no alternatives to the study of pathogenesis and vaccine responses to these viruses, we have considered the options of using cell lines derived from the tumours as part of principle of Replacement of animal usage for studying some of the aspects of virus-host interactions. For example, we are using gene editing approaches on avian oncogenic virus-transformed cell lines to examine the effects of knockout of oncogenes such as the Meq, c-myc and v-rel as well as miRNAs such as miR-155 and miR-17-92 cluster. However, these alone could not provide all the information as these may not be similar to the interactions in the primary tumours, and also may not be sufficient to know the pathways that trigger neoplastic transformation. Similarly, there are no in vitro alternatives for studying the

immune responses to these viruses and vaccines, although we are exploring possibilities of using organoids (organ on a chip) for certain studies.