COMMERCIAL IN CONFIDENCE

DEVELOPMENT OF A LIVE ATTENUATED VACCINE FOR CHICKEN ANAEMIA VIRUS

RIRDC Project No. UM-37A

FINAL REPORT

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Foreword

The role of Chicken Anaemia Virus (CAV) in immunosuppressive diseases has become more evident over recent years. It contributes not only to severe immunosuppression in progeny from recently exposed parents, but also to high MDV mortalities, associated with donor progeny susceptible to CAV, both in Australia and overseas. CAV has frequently been isolated from affected birds in ultravirulent MDV outbreaks.

Increasing attention is now being paid to declining immunity in donor flock dams which result in variable to poor progeny performance. A similar situation has been identified for CAV to that pertaining to Infectious Bursal Disease Virus in the past - the importance of maintaining a good level of immunity in donor flocks producing hatching eggs.

In Australia and elsewhere in the world there is no attenuated CAV vaccine available. Fully virulent CAV has been used for controlled exposure, but while this has been successful for clinical disease in some situations, such methods perpetuate the presence of virulent CAV in the environment, contribute to subclinical disease, and are not available throughout the industry. The problem of declining immunity in donor flocks is not addressed by this method because of the risk of exposing subpopulations of birds within donor flocks that are susceptible to reinfection with CAV and hence contributing to vertical transmission. The need for a safe immunogenic, universally available attenuated CAV vaccine is clearly evident.

The aim of this project has been to produce a clone of the virus amenable to manipulation, to develop methods for site specific attenuation of CAV, and then to commence assessment of mutants generated using these methods, with the ultimate goal of development of an attenuated strain of CAV suitable for general vaccination of poultry flocks.

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Contents

Executive Summary

Chicken Anaemia Virus (CAV) is a virus whose role in immunosuppressive diseases has become more evident over recent years. It has now been found to be causally associated with high Marek's disease mortalities, with progeny from poorly CAV-immune parents showing exacerbated MDV mortalities both in Australia and overseas. CAV has frequently been isolated from affected birds in ultravirulent MDV outbreaks. Increasing attention is now being paid to declining immunity in donor flock dams which result in variable to poor progeny performance. The importance of maintaining a good level of immunity in donor flocks producing hatching eggs is similar to the situation seen with Infectious Bursal Disease Virus in the past. Currently in Australia there is no attenuated CAV vaccine available. Fully virulent CAV has been used in a controlled exposure protocol, but while this has been successful, it perpetuates the presence of virulent CAV in the environment. The problem of declining immunity in donor flocks is not addressed by this approach because of the risk of exposing subpopulations of birds within donor flocks that are susceptible to reinfection with CAV and hence vertical transmission. The move to cage rearing is likely to increase the risk of clinical CAV infections as cage-reared birds are less likely to be uniformly exposed and thus less likely to have developed immunity by the time they commence production. The need for a safe immunogenic attenuated CAV vaccine is clearly evident. Improved control of CAV is also likely to result in reduced need for medication of flocks to control secondary diseases, and thus would result in benefits in environmental sustainability and public health.

This project has made considerable progress towards developing a defined attenuated CAV vaccine strain. We have developed an infectious genomic clone of a virulent Australian strain of CAV and produced a series of 16 defined mutants of CAV. Of the 16 mutants six have been shown to be unable to replicate, but seven of the other ten replicate *in vitro*. Preliminary work has indicated that six of these mutants are attenuated to some extent in chick embryos, with four sufficiently attenuated to suggest they may be useful vaccine strains. A further two years work is necessary to enable a full assessment of the potential of the mutants developed thus far. This will enable us to determine their capacity to induce an immune response protective against challenge with virulent CAV and, if required, to further mutate the current mutants to increase the degree of attenuation of the vaccine strain and to reduce the risk of reversion of these strains to virulence.

Introduction

CAV is a significant cause of loss in the poultry industry, particularly due to its enhancement of disease caused by Marek's disease virus, but also through diminished efficacy of vaccination to control other diseases. The considerable resistance of the virus to heat and disinfectants, as well as its capacity to spread both horizontally and vertically compounds the difficulties of controlling its effects. With the increasing requirement to improve biosecurity to control Marek's disease, the significance of CAV can be predicted to increase, as in the absence of suitable vaccines, more parent flocks will come into lay without having encountered CAV, and hence their progeny will not be protected. Current control measures rely on infection of parent birds with a virulent strain before they come into lay, or on natural exposure (encouraged by maintaining a "dirty system"), but have the major disadvantage that this perpetuates a high level of contamination of parent operations with virulent virus. Furthermore the potential problems due to waning levels of immunity in older birds (and hence reduced passively acquired protection in their chicks) cannot be adequately addressed by reexposure to virulent virus without risking vertical transmission to some of the flock. It is clear that an attenuated live vaccine, with boosting of parent flocks using an inactivated vaccine, would be a preferable alternative to the current measures.

Cost of CAV Infections to the Poultry Industry

The cost of CAV infections to the poultry industry in Australia have not been estimated. However studies overseas in broilers have estimated the losses due to clinical CAV infections to be between 3.3% to 12.8% depression in average bird weight with a greater proportion of the flock falling into lighter weight bands, and also 2% higher mortalities than in unaffected flocks. In studies of flocks which did not show clinical signs of disease, but had developed active immunity to CAV by the time of slaughter, feed conversion ratios were reduced by 2%, average bird weight was reduced by 2.5%, and net income per bird was reduced by 13%. In addition to these losses, there is a significant contribution by CAV to the more severe outbreaks of Marek's disease which are currently a major concern to the Australian industry. In addition to the well established potentiation of Marek's disease, CAV can also aggravate infections with infectious bursal disease, lentogenic Newcastle disease, reticuloendotheliosis virus and other bacterial and viral pathogens.

Expected Benefits of Research to Industry

The expected result of this project, generation of an attenuated strain of CAV suitable for vaccination of chickens, will assist in the control of losses due to CAV in both broiler and layer flocks. Current use of virulent strains of CAV, while effective against clinical disease, is ensuring continuing subclinical infection of birds and, on the basis of studies overseas, is probably resulting in ongoing significant losses. In addition, improved control of CAV will contribute to improved efficacy of vaccination against other infectious diseases, including Marek's disease.

Environmental Benefits

Improved control of CAV will result in reduced levels of secondary infections with bacterial pathogens, and hence a reduced need for treatment of poultry with antimicrobial drugs. Reduction of antimicrobial usage has significant environmental and public health benefits.

Consultation with Poultry Industry and Advisors

In consultation with industry advisors, two issues have become apparent. Firstly current control measures are not universally available to the whole industry, and thus some producers are at a distinct disadvantage, and secondly the virulent strains currently in use are unlikely to be registerable, and hence will have a limited future. This advice suggests that improved CAV control is a significant issue for the whole industry. There also continues to be considerable industry concern over the reduced capacity of current Marek's disease vaccines to adequately control problems, necessitating a reevaluation of approaches to control this disease. While improved Marek's vaccines are one goal, increased biosecurity and control of synergistic infections are likely to be just as important in achieving effective control.

Dissemination and Commercialisation of Results

This laboratory has a strong record of collaboration with both the poultry industries and the veterinary biological industries, and has been able to rapidly transfer research into solutions for the poultry industries as a result. This has included the rapid commercial release of mycoplasma vaccines and diagnostic reagents. Several national and international biological manufacturers have expressed

interest in commercialisation of attenuated CAV vaccines, and use of such a commercial collaborator for release of the vaccine resulting from this project will ensure universal availability of the vaccine to the industry.

Chicken anaemia virus was first isolated by Yuasa *et al* in 1979 in day old chicks (3) . Subsequently the MDCC-MSB1 cell line, which is derived from Marek's disease virus induced lymphomas, was used for propagation (4) . Other cell lines have been used, but all are cells transformed by either Marek's disease virus or avian leukosis virus. As a result cell culture propagated virus is not suitable for use in vaccines due to contamination by these viruses. Currently commercial vaccines need to be produced in chick embryos from CAV free flocks. Australia is in a privileged position through the availability of CAV-free specific pathogen free birds for both experimental work and for the production of vaccines.

CAV is very resistant to inactivation by heat, surviving temperatures of 70°C for up to an hour (3) . In addition it is resistant to many disinfectants, including quaternary ammonium compounds, iodophors and formalin, although it is destroyed by hypochlorite (1) .

Disease

The disease caused by CAV in experimental infections is characterised by infection and destruction of haemocytoblast (red cell lines) and cortical thymocyte (cytotoxic and helper lymphocyte) cells, resulting in severe anaemia and immunodeficiency (3, 5, 6) . Overt disease is only apparent in birds under 3 weeks of age, but older birds can still be infected, and significant losses in production have been observed in subclinically affected broiler flocks which had seroconverted by slaughter (3, 5-7). Experimental studies have shown that while anaemia is not apparent in orally infected 3 week old birds, they still have significant depression of their cell mediated immune function for at least 4 weeks after infection, thus potentially explaining the basis of the subclinical losses seen in flocks which are infected after maternal immunity wanes (8, 9). The disease seen in the field is more complex than that seen in experimental infections and includes higher rates of other diseases such as colibacillosis, more severe manifestations of Marek's disease, and a variety of opportunistic infections (5). The interaction between CAV and Marek's disease virus (10) is of particular concern given the increasing problems with control of Marek's disease in Australia.

Epidemiology

CAV occurs worldwide and the primary host appears to be the chicken. The prevalence of infection in older flocks is high and a number of workers have also reported a high incidence of infection in broiler flocks by the time of slaughter (11-14). The virus is transmitted both vertically and horizontally, with most outbreaks linked to vertical transmission from an acutely infected parent flock, followed by horizontal transmission among the progeny. Maternal antibody protects against CAV infection for about 3 weeks after hatch, but while most parent flocks seroconvert between 8 and 12 weeks of age, some flocks do not seroconvert until they come into lay. Infection at this stage results in clinical disease in progeny for 3 to 6 weeks.

Experimental Reproduction of Disease

Experimental reproduction of disease due to CAV has been achieved by dosing day old chicks orally with 10^5 TCID50 of virus, with 25% of birds developing anaemia (9, 15). Age resistance to disease develops by 2 weeks of age, although infection is still possible . Parenteral inoculation of day old chicks results in 100% of birds with anaemia (3, 5, 16). In addition these birds have reduced bursa weights and body weights. More severe disease can be induced by infection of chick embryos via the yolk sac, with chicks dying at 10-15 days of age (17).

Control Measures

Current control measures for CAV rely on the use of virulent CAV to vaccinate parent chickens before they come into lay. While this has been effective in eliminating most clinical disease, there are four areas of concern in relying on this approach. The first is the waning levels of maternal immunity provided by older flocks (18). Use of virulent strains of CAV for boosting birds in lay carries the risk that some parents will transmit the virus to their progeny, and thus cause outbreaks of clinical disease. The second concern is that ongoing use of virulent CAV perpetuates a cycle which ensures high levels of environmental challenge by CAV for all birds. Such high levels of challenge may well contribute to vaccine breakdowns. Thirdly the data from numerous sources suggests that subclinical CAV infection has adverse effects on feed conversion ratios, bird weight and net income (7, 19). Where virulent vaccine strains are in use they are likely to be the major source of subclinical

infections in broilers, and hence a cause of significant loss. Finally the synergism between CAV and Marek's disease virus suggests that ongoing high levels of challenge by virulent CAV is proably contributing to the increasing difficulties experienced in control of Marek's disease. There is a clear requirement for an attenuated vaccine strain of CAV to enhance control and reduce the losses inevitably associated with continuing use of virulent strains for control of clinical disease.

Problems in Producing a Vaccine

There have been attempts to produce attenuated strains of CAV using traditional approaches. Extended passage of CAV in cell culture reduced, but did not eliminate, the pathogenicity of CAV isolates. In addition strains produced by such methods have rapidly reverted to virulence on repeated passage in chickens (20). The possibility of developing inactivated vaccines is restricted by the inability to produce high titred virus in either cell culture or chick embryos.

Three alternative approaches are possible. The first is to produce subunit vaccines or virus-like particles by expressing CAV proteins in baculoviruses (viruses which infect insect cells and which can be used to engineer high levels of production of recombinant proteins). This approach would focus on the simultaneous production of virus protein 1 (VP1 - the capsid protein) and virus protein 2 (VP2) in the same cell, as this appears to be necessary to produce protein which can induce neutralising antibody (21). This approach may be suitable for production of a vaccine for administration to parent birds, but because of costs of delivery would not be appropriate for administration to broilers or layer pullets.

The second approach is to exploit the small genome size of CAV to generate mutants which are suitable for use as attenuated vaccines. These vaccines could be produced in chick embryos and administered in drinking water for immunisation of parents, broilers and pullets.

Finally attenuated mutants of CAV could be used in a more novel approach. The full length genome of CAV is infectious in its own right. The genome alone can be transfected into cell lines to produce infectious virus (20, 22-25). This capacity could be exploited by adopting the relatively new technique of DNA vaccination. DNA alone could be used for *in ovo* vaccination, with uptake by the cells of the embryo resulting in the eventual production of the vaccinating virus. Given the increased virulence of CAV for embryos it is essential that the genome used be attenuated. However molecular biological techniques could be used to produce large quantities of genomic DNA very cheaply, thus avoiding the problems associated with contaminated cell lines and low titres of infectious virus. Such a DNA vaccine could also be used intramuscularly in older birds to boost parents to prevent the occurrence of the old parent syndrome, thus effectively acting as a killed vaccine.

Molecular Biology of CAV

The reason a directed mutation approach is feasible in CAV is its small and relatively simple genome. At 2,300 base pairs it has one of the smallest of all viral genomes, and this genome can be amplified in its entirety using the polymerase chain reaction (PCR). The isolated linear genome can be transfected into cell lines to reproduce infectious virus (20, 22-25). Thus manipulation of the genome to produce site specific mutations is achievable. The virus produces 3 known proteins VP1, VP2 and VP3. VP3 (also known as apoptin) has been shown to be capable of causing apoptosis (cell death) in cells by itself (26). VP1 appears to be the capsid protein. There is a single viral promoter with a series of enhancer elements (27).

Objectives

•To improve control of CAV related disease in chickens.

•To perform directed mutagenesis on the genome of chicken anaemia virus (CAV) with the aim of developing attenuated mutants

•To develop and assess attenuated mutants of CAV for their suitability as live vaccines for administration to parent flocks, broilers and layer pullets

•Assess the suitability of DNA vaccination with the genome of these attenuated mutants

Methodology

Characterisation of a virulent Australian CAV isolate

The CAV isolate used (CAU269/7) was originally isolated from a commercial breeder flock in Australia. The virus was cultured in the Marek’s disease virus transformed lymphoblastoid cell line, MDCC-MSB1. Inoculation of CAU269/7 into MDCC-MSB1 cells produced cytopathic effects

consistent with that of other reported CAV isolates and was characterised by the appearance of enlarged, misshapen cells within 40 h. Total cell degeneration was apparent within 96 h after infection. Infected cells were stained for the CAV-specific protein, VP3, in an indirect immunofluorescence assay (IFA) using the mouse derived monoclonal antibody JCU/CAV/1C1 (JCU TropBio, Townsville, Queensland). Infected cells showed strong fluorescence relative to mock-infected control cells. To assess the pathogenicity of the Australian isolate CAU269/7 for chickens, culture medium (~$1x10^{5.5}$ TCID50 /mL) from CAV-infected cells was used to inoculate yolk sacs of fifteen CAV-free, 7-day-old, specific-pathogen-free (SPF) chicken embryos (SPAFAS, Parkville, Victoria). In parallel, fifteen 7-day-old chicken embryos were mock-infected with CAV-free culture medium. Thirteen days postinoculation (day 20; 1 day before hatch), five embryos from each group were euthanased and samples of heparinised peripheral blood were collected. No significant difference in packed cell volumes (PCV) were observed between the groups. Eight days later (day 7 posthatching), the PCV and total body weights of all remaining chicks were determined. At this time, infected chickens (n = 9; one dead from causes unrelated to CAV) had lower body weights ($P = 0.049$) and lower PCVs ($P < 0.001$) than the control group. All remaining chicks were euthanased at day 12 after hatching and samples of bone marrow were collected for analysis. Infected chicks (n = 5; four dead from CAV related infection) exhibited clinical signs associated with infection with CAV, including weakness, depression, anorexia, stunted growth, low PCV, pale bone marrow, thymus atrophy and haemorrhages in the subcutaneous tissues and skeletal muscles. Body weights of infected chicks were significantly lower ($P < 0.001$) than those of uninfected chicks in the control group, which developed none of the clinical signs described above. In addition, CAV-infected cells, identified by IFA for VP3, were seen in bone marrow smears.

Construction of a clone of the genome of an Australian strain of CAV.

To clone the genome of isolate CAU269/7, total DNA was isolated from CAV-infected MDCC-MSB1 cultures at 60 h postinfection using a proteinase K/SDS digestion and phenol/chloroform extraction protocol. For polymerase chain reaction (PCR) amplification of the CAV genome two pairs of oligonucleotide primers (C1 - 5' CTATCGAATTCCGAGTGGTTACTAT 3' and C7 -5' CTTATTTGTGCCTTGCGCTA 3', C5 - 5' CAGTTTCTAGACGGTCCTTC 3' and C25 - 5' CGGAATTCGATTGTGCGGTGAACG 3') were synthesised. The sequences of these primers were based on the DNA sequence of the German CAECUX-1 CAV isolate. Primer pairs (C1+C7 and C5+C25) were used to generate two overlapping PCR products that covered the entire 2.3 kb CAV genome. These were digested with *Eco*RI and *Xba*I restriction endonucleases and ligated into pGEM-4Z (Promega, Sydney) that had been digested with the same enzymes to derive a plasmid containing the full-length genome of CAU269/7, designated pCAU269/7. *Eco*RI restriction endonuclease sites were introduced at either end of the genome by PCR mutagenesis to facilitate release of the complete CAV genome from the plasmid vector. This site was not present in CAU269/7 but does occur in other isolates at this position. The DNA sequence of CAU269/7 was determined using a combination of vector specific (T7 and SP6) and CAV sequence specific primers. The complete sequence of each DNA strand, with the exception of a short region which could not be resolved, was determined at least twice and submitted to GenBank (Accession No. AF227982).

Establishing infectivity of the cloned CAV genome

In order to determine the infectious nature of the cloned CAV DNA, the 2.3 kb CAV genomic fragment was released from pCAU269/7 by *Eco*RI digestion and transfected by electroporation into MDCC-MSB1 cells either in a linear form or as closed circular (re-ligated) DNA. Briefly, $4x10^6$ cells were washed twice with sterile PBS and resuspended in 700 µL PBS containing 10 µg DNA. Cells were pulsed with 400 V/375 µF then immediately transferred to 4.5 mL pre-warmed culture medium containing 10% foetal calf serum.

Site Directed Mutagenesis of the CAV Genome

Oversewing PCR mutagenesis has been used to introduce single or multiple mutations into the infectious cloned CAV genome pCAU269/7. The mutations we have introduced have had two specific aims. The first has been to introduce mutations that can be predicted to alter the rate of production of the viral proteins during infection, and the second has been to alter the function of the viral proteins.

To alter the rate of production of viral proteins we have introduced mutations into the region that is predicted to be responsible for initiation of translation of the first of the viral proteins, VP2. In the

wild type virus this region is predicted to be relatively inefficient in the initiation of translation, and hence it is possible that the second of the viral proteins, VP3, is translated by ribosomes that scan past this first, inefficient, translational initiation region and then commence translation at the region that immediately precedes VP3. Thus mutagenesis was used to create a more efficient translational initiation region preceding VP2, expecting that this would reduce production of VP3 (apoptin), the protein shown to induce cell death in CAV infected cells.

As work elsewhere has focussed on the function of VP3, we have focussed our attempts to disrupt function on the poorly understood viral protein VP2 and on the capsid protein VP1. The mutations we have introduced into these proteins have been designed to disrupt regions that we predict to be important in protein function. Our examination of the sequence of VP2 suggested that it may have at least 4 critical regions. The first we predict to be involved in an enzymatic function of the protein (a predicted protein tyrosine phosphatase function) and the other three, two acidic alpha helical regions separated by a basic beta sheet region, we predict to be involved in DNA binding. In VP1 we have focussed mutagenesis on a region we have predicted to be involved in DNA binding.

Assessment of the Attenuation of CAV by Site Directed Mutation

As site directed mutations in the cloned CAV genome have been produced they have been assessed in three ways. Initial assessment uses transfection into MDCC-MSB1 cells, using the technique described in detail above, to determine whether the mutation still allows viral replication. After transfection the MDCC-MSB1 cells are passaged at least 10 times and viral replication is assessed by IFA staining of the cells. If viral replication appears to be occurring in the transfected cells (that is the number of IFA positive cells increases with passage), stocks are made of the virus, and PCR and DNA sequencing are used to confirm that the virus replicating in the cells has sequence identical to the cloned, mutagenised genome used for tranfection. The growth curve of the mutant virus is then examined, and the phenotype of the infected cells is examined by immunofluorescence.

As CAV is most pathogenic in embryos, we are next assessing the viable mutants by inoculation of embryos with virus obtained from transfected MDCC-MSB1 cells. We are assessing infected embryos at 20 days of incubation as well as after hatching.

Development of *in vitro* models for assessing attenuating mutations

While assessment of mutants by examining their behaviour after transfection is feasible, it does require an extended period of time to assess each mutant. We have thus attempted to develop methods that enable the effect of mutants to be assessed more rapidly. To assess mutants affecting the initiation of translation of the three genes, a series of DNA constructs were produced with the chloramphenicol acetyl transferase (CAT) gene introduced into the cloned CAV genome immediately after the start codon of each of the three genes. The production of CAT can be detected rapidly, quantitatively and very sensitively by examining acetylation of chloramphenicol using thin layer chromatography. Mutations can then be introduced into the region predicted to control initiation of translation and the effect these mutations are likely to have on viral translation of proteins assessed by transfecting the mutated construct into MDCC-MSB1 cells and measuring CAT activity.

To assess the effect of mutation on VP2 function we have introduced the gene for VP2 into an expression vector and produced purified VP2 in large quantities. We have predicted that VP2 may have protein tyrosine phosphatase activity based on genetic database comparisons. This activity, if present, is likely to have a key role in disruption of signalling pathways in the infected cell and thus a major role in the pathogenesis of CAV infections. To confirm this predicted activity we have been assessing the capacity of purified VP2 to dephosphorylate a synthetic peptide containing tyrosine phosphate. Dephosphorylation of the peptide has been detected using a malachite green assay to measure the amount of free phosphate produced. This assay can then be used to rapidly examine purified VP2 with mutations introduced into the regions predicted to be involved in the enzymatic function and specific degrees of reduction in the enzymatic function correlated with specific mutations, thus allowing a closer definition of the disruption of VP2 function caused by mutation.

Detailed Results

The strain of CAV which we are working with was found to contain a restriction endonuclease cleavage site which prevented further development without mutagenesis to remove the site. This site has now been removed by mutagenesis and the mutagenised virus genome has been transfected into

cells to assess its suitability for further work. The need to do this has delayed development of mutants. . It was originally proposed that site-directed mutagenesis by PCR would be conducted on a clone consisting of two head-to-tail (tandem) repeats of the full CAV genome in a single plasmid vector. A newer, simpler method was adopted for site-directed mutagenesis of the CAV genome. This method required only a single copy of the CAV genome to be cloned into a plasmid vector. This approach enabled site-directed mutagenesis to be performed at any site on the CAV genome.

Characterisation of the cloned CAV genome

The organisation of the genome of Australian CAV isolate CAU269/7 was similar to other reported CAV isolates. It is composed of 2298 nucleotides and contains three overlapping open reading frames (ORFs) encoding the VP1, VP2 and VP3 proteins that have been identified in other CAV isolates. CAU269/7 contained four almost-perfect 21 bp direct repeat sequences (nucleotides [nt] 144-237) upstream of a single transcriptional start site (position +1 in Figure 1A) and a polyadenylation signal (nt 2266). The small noncoding region of the CAV genome (nt 2185-2251) possesses a high GC content and contains sequences considered to be involved in the termination of transcription. Others have reported difficulties in resolving the sequence in this region using standard chain termination sequencing chemistries. We also experienced difficulties in determining the sequence of this region, despite the use of a range of standard approaches designed to resolve such sequences, and were unable to confirm the identity of 12 residues (nt 2193-2204). These residues do not vary between the known CAV isolates and so for the purposes of the analysis presented herein we have inserted the corresponding CAECUX-1 sequence into this region of CAU269/7. CAU269/7 demonstrated an overall nucleotide sequence identity of approximately 95% with each of the seven other sequenced CAV isolates, indicating that this isolate was closely related to, but clearly distinct from, other isolates. This was a lower identity than any of the other isolates have with each other (96-99%). The amino acid identities of CAU269/7 with other sequenced isolates ranged from 96-99%, 98-99% and 94- 98% for VP1, VP2 and VP3 proteins, respectively. The majority of nucleotide differences between the isolates were synonymous. Most of the non-synonymous mutations, particularly in VP2 and VP3, were not consistently variable across isolates (data not shown). One 13-amino-acid region within VP1 (residues 139-151) contained a cluster of non-synonymous changes that did vary between isolates. Naturally occurring amino acid changes at positions 139 and 144 have been shown to markedly affect the growth and spread of CAV isolates in cultured cells highlighting a possible functional role for the hypervariable region. Residue 144 displays remarkable variability with four different amino acids found in this position. It is likely that variability observed within residues 139-151 is a consequence of its biological significance and reflects the selection of variants in response to environmental/host cell changes and/or immune selection. To more closely examine the evolutionary relationships of CAV isolates, a phylogenetic analysis was performed. While CAU269/7 is phylogenetically distinct from all other isolates, it appeared to be most closely related to another Australian isolate (CAU65414). Interestingly, these two Australian isolates have different sequences in the hypervariable region of VP1. This analysis also showed that CAU269/7 is more similar to the Japanese isolate than to those originating in Europe or the USA.

Establishing infectivity of the cloned CAV genome

Transfection of MDCC-MSB1 cells with the *Eco*RI-released insert of plasmid clone pCAU269/7 resulted in patterns of infection similar to those observed in virion-initiated infections. Usually 1 to 2 passages of transfected cells were required before virus-induced cell death was apparent. At several time points after transfection, cells were analysed for the presence of CAV by IFA. Cells transfected with EcoRI-digested pCAU269/7 reacted with the anti-VP3 monoclonal antibody, while cells transfected with control DNA, consisting of a truncated form of the full-length CAV genome, did not. Furthermore, medium from pCAU269/7 transfected cultures passaged onto fresh MDCC-MSB1 cultures produced cytopathic effects characteristic of CAV infection. Taken together, these data show that pCAU269/7 contains all the elements necessary for generating CAV infection in MDCC-MSB1 culture.

Site specific mutants of CAV

We have now derived a large series of mutants altered at specific sites in four areas of the CAV genome. The first series of four mutants were predicted to influence the efficiency of translation of the VP3 and VP2 proteins, which are thought to control viral virulence. The aim was to reduce the

quantity of these proteins produced during infection. All 4 mutants have been introduced into cells but only one was viable. This mutant only appears to be viable for a limited time, with some viral replication initially, but eventual loss from the cells. It thus appears unlikely that these mutants will produce an attenuated strain suitable for administration to chickens. It is possible that a mutant with limited capacity to replicate might make a suitable DNA vaccine for administration *in ovo*. We have developed the *in vitro* model using CAT as a reporter gene to assess additional mutations in these translational control regions, and should we produce any further mutants in this region we will be able to establish the effects they have in this *in vitro* assay, which should speed the characterisation.

The second series of mutants have been focussed on the VP2 gene. We have generated 10 mutants in the coding region of the gene, attempting to disrupt the specific structural features we have predicted to be important in the function of the protein. We have six mutants affecting the amino acids predicted to be crucial for the protein tyrosine phosphatase activity, four mutants disrupting the basic beta sheet region of the protein by either changing basic residues to neutral residues, or by making changes likely to cause disruption to the formation of the helix, and most recently have produced three mutants affecting the acidic regions of VP2. We have also commenced producing mutations in the basic helical region of VP1. All the mutants with changes in the enzymatic region and in the basic region have been transfected into cells and preliminary examination indicates that all are viable. At least two are capable of replication to sufficient titres to be viable for vaccine production. Examination of the phenotypes of these mutants *in vitro* has shown that their patterns of replication have been altered by the mutations, with an altered pattern of distribution of the VP3 protein in infected cells, a slower rate of growth *in vitro* and in one mutant a significant decrease in viral release from infected cells. These *in vitro* phenotypes suggest that they are likely to be attenuated *in vivo*.

We have commenced assessment of these mutants by infection of 7 day old embryos. Preliminary assessment of the initial results with six of the mutants suggests that all are attenuated to some extent compared to the parent virus, with four significantly attenuated.

The *in vitro* assay of protein tyrosine phosphatase activity has been developed and our preliminary results suggest that VP2 is a potent protein tyrosine phosphatase. We are now assessing the activity of seven mutated versions of VP2 in this assay to establish a correlation between this activity and attenuation. Once a correlation is established it will be possible to titrate the desired amount of attenuation introduced by mutations by assessing their effects in these *in vitro* assays.

DNA vaccination

Preliminary evidence of the potential of DNA vaccination has been obtained using a clone of the CAV genome in 6 day old embryos. There was some evidence that birds inoculated as embryos with this defective CAV genome were protected after hatching from the effects of CAV infection. These studies need to be repeated to confirm these observations and to assess whether more effective protection can be achieved with CAV genomes that are capable of replication.

Discussion of Results

We have established a method to generate CAV strains with defined mutations and have developed methods to allow preliminary assessment of mutants *in vitro*. A series of mutants have been produced and *in vitro* assessments of many of these mutants have been completed. Mutations affecting the efficiency of translation of the two viral proteins likely to be most responsible for virulence prevented replication of the virus and thus are not likely to be useful for production of an attenuated vaccine. However mutations in the coding region of VP2 resulted in viruses that could still replicate in cell cultures and in embryos, but which, on preliminary observations, appear to be attenuated to some extent. Further assessment is needed to characterise the extent of attenuation that has been effected by these mutations, and it may be necessary to combine some of the mutations to produce a strain that is sufficiently attenuated to be suitable as a vaccine strain.

Further work is also needed with these mutants to establish whether the absence of severe gross lesions in embryos correlates with absence of immunosuppression in chicks and whether they induce good protective immunity in inoculated birds.

Implications

The feasibility of using site specific mutagenesis to produce attenuated strains of CAV has been demonstrated. Furthermore some vaccine candidates have been produced. Further work is needed to assess the extent of attenuation achieved, and additional mutagenesis may be needed to produce a strain that is suitable for use as a vaccine. Work still needs to be done to assess protective immunity induced by these strains, and further work is needed to assess the potential of DNA vaccination.

Dissemination/Adoption

Preliminary results have been presented to the Australian Veterinary Poultry Association meeting. Once a candidate strain has been shown to induce protective immunity interest in development and protection of intellectual property will be sought from biologicals manufacturers, several of whom have already expressed interest in commercialisation of any vaccine arising from the project.

Publications

BROWN, H. K., BROWNING, G. F., SCOTT, P. C., AND CRABB, B. S. – Full-length infectious clone of a pathogenic Australian isolate of chicken anaemia virus. Australian Veterinary Journal, 78:637-640 (2000).

Recommendations

The approach developed in this project appears likely to result in attenuated strains of CAV. These strains will need to be examined to confirm their attenuation and to assess their capacity to induce adequate protective immunity. Such studies are likely to require a further two years work. By this stage the intellectual property generated by the project is likely to be sufficiently advanced to attract support from biologicals manufacturers to take the vaccine through the field trial stage and the registration process.

Intellectual Property

Protection of the intellectual property from this project will be sought, by filing a provisional patent application, once there is conclusive evidence that the mutants that have been developed are attenuated for chicks.

Communications Strategy

Once definitive evidence of attenuation and protective immunity has been obtained, and intellectual property has been protected, the results of the project will be presented at international meetings and biologicals manufacturers will be approached directly to assess their interest in pursuing commercialisation. A number of local and international biological manufacturers have expressed interest in commercialisation of attenuated CAV vaccines, and use of such a commercial collaborator for release of the vaccine resulting from this project will ensure universal availability of the vaccine to the industry. The production of a vaccine strain will also be communicated to the industry through the Australian Veterinary Poultry Association meetings.

Bibliography

1. McNulty MS. Chicken anaemia agent: a review. Avian Pathology 1991;20(2):187-203.
2. Noteborn M, Koch G. Chicken anaemia virus infection - molecular basis of pathogenicity. Avian Pathology 1995;24(1):11-31.
3. Yuasa N, Taniguchi T, Yoshida I. Isolation and characteristics of an agent inducing anemia in chicks. Avian Dis 1979;23:366-385.
4. Yuasa N, Taniguchi T, Goda M, Shibatani M, Imada T, Hihara H. Isolation of chicken anemia agent with MDCC-MSB1 cells from chickens in the field. National Institute of Animal Health Quarterly, Japan 1983;23(3):75-77.
5. Otaki Y, Nunoya T, Tajima M, Tamada H, Nomura Y. Isolation of chicken anaemia agent and Marek's disease virus from chickens vaccinated with turkey herpesvirus and lesions induced in chicks by inoculating both agents. Avian Pathology 1987;16(2):291-306.
6. Goryo M, Suwa T, Umemura T, Itakura C, Yamashiro S. Histopathology of chicks inoculated with chicken anaemia agent (MSB1-TK5803 strain). Avian Pathology 1989;18(1):73-89.

7. McNulty MS, McIlroy SG, Bruce DW, Todd D. Economic effects of subclinical chicken anemia agent infection in broiler chickens. Avian Dis 1991;35(2):263-8.
8. McConnell CD, Adair BM, McNulty MS. Effects of chicken anemia virus on macrophage function in chickens. Avian Dis 1993;37(2):358-65.
9. McConnell CD, Adair BM, McNulty MS. Effects of chicken anemia virus on cell-mediated immune function in chickens exposed to the virus by a natural route. Avian Dis 1993;37(2):366-74.
10. Jeurissen SH, de Boer GF. Chicken anaemia virus influences the pathogenesis of Marek's disease in experimental infections, depending on the dose of Marek's disease virus. Vet Q 1993;15(3):81-4.
11. Rosenberger JK, Cloud SS. The isolation and characterization of chicken anemia agent (CAA) from broilers in the United States. Avian Diseases 1989;33(4):707-713.
12. McNulty MS, Connor TJ, McNeilly F, Kirkpatrick KS, McFerran JB. A serological survey of domestic poultry in the United Kingdom for antibody to chicken anaemia agent. Avian Pathology 1988;17(2):315-324.
13. von Bulow V. Unsatisfactory sensitivity and specificity of indirect immunofluorescence tests for the presence or absence of antibodies to chicken anaemia agent (CAA) in sera of SPF and broiler breeder chickens. Journal of Veterinary Medicine B 1988;35(8):594-600.
14. Lucio B, Schat KA, Shivaprasad HL. Identification of the chicken anemia agent, reproduction of the disease, and serological survey in the United States. Avian Diseases 1990;34(1):146-153.
15. McNeilly F, Smyth JA, Adair BM, McNulty MS. Synergism between chicken anemia virus (CAV) and avian reovirus following dual infection of 1-day-old chicks by a natural route. Avian Diseases 1995;39(3):532-537.
16. Goryo M, Sugimura H, Matsumoto S, Umemura T, Itakura C. Isolation of an agent inducing chicken anaemia. Avian Pathology 1985;14(4):483-496.
17. Yuasa N, Yoshida I. Experimental egg transmission of chicken anemia agent. National Institute of Animal Health Quarterly, Japan 1983;23(3):99-100.
18. Imai K, Mase S, Tsukamoto K, Hihara H, Matsumura T, Yuasa N. A long term observation of antibody status to chicken anaemia virus in individual chickens of breeder flocks. Res Vet Sci 1993;54(3):392-6.
19. McIlroy SG, McNulty MS, Bruce DW, Smyth JA, Goodall EA, Alcorn MJ. Economic effects of clinical chicken anemia agent infection on profitable broiler production. Avian Dis 1992;36(3):566-74.
20. Todd D, Connor TJ, Calvert VM, Creelan JL, Meehan BM, Mcnulty MS. Molecular cloning of an attenuated chicken anaemia virus isolate following repeated cell culture passage. Avian Pathology 1995;24(1):171-187.
21. Koch G, van Roozelaar D, Verschueren CA, van der Eb AJ, Noteborn MH. Immunogenic and protective properties of chicken anaemia virus proteins expressed by baculovirus. Vaccine 1995;13(8):763-70.
22. Claessens JA, Schrier CC, Mockett AP, Jagt EH, Sondermeijer PJ. Molecular cloning and sequence analysis of the genome of chicken anaemia agent. J Gen Virol 1991;.
23. Meehan BM, Todd D, Creelan JL, Earle JA, Hoey EM, McNulty MS. Characterization of viral DNAs from cells infected with chicken anaemia agent: sequence analysis of the cloned replicative form and transfection capabilities of cloned genome fragments. Arch Virol 1992;124(3-4):301-19.
24. Noteborn M, de Boer G, van Roozelaar D, et al. Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. Journal of Virology 1991;65(6):3131-3139.
25. Todd D, Creelan JL, Meehan BM, Mcnulty MS. Investigation of the transfection capability of cloned tandemly-repeated chicken anaemia virus DNA fragments. Archives of Virology 1996;141(8):1523-1534.
26. Noteborn MH, Todd D, Verschueren CA, et al. A single chicken anemia virus protein induces apoptosis. J Virol 1994;68(1):346-51.
27. Noteborn MH, Verschueren CA, Zantema A, Koch G, van der Eb AJ. Identification of the promoter region of chicken anemia virus (CAV) containing a novel enhancer-like element. Gene 1994;150(2):313-8.