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Evaluation of efficacy of mammalian and baculovirus expressed E2 subunit vaccine candidates to bovine viral diarrhoea virus

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) is a worldwide pathogen of cattle causing a wide spectrum of clinical disease. The major envelope glycoprotein of BVDV, E2, induces the production of neutralising antibodies. In this study we compared the protection afforded to cattle after BVDV challenge by two separate E2 vaccine candidates produced by different heterologous protein expression systems. E2 antigen was expressed using the baculovirus expression system (brE2) and a mammalian cell expression system (mrE2). In the first vaccination study the quantity of recombinant protein expressed by the two systems differed. Vaccination of cattle with a higher dose of brE2 or low dose mrE2 gave comparable protection from viral challenge. Immunised animals showed no pyrexia and reduced leucopaenia which contrasted to the unvaccinated controls. In addition virus shedding from the nasal mucosa was decreased in the vaccinated groups and strong humoral responses were evident post-challenge. However, the efficacy of the brE2 vaccine was greatly diminished when a reduced dose was tested, indicating the importance of assessing the type of expression system used in antigen production.

1. Introduction

Bovine viral diarrhoea virus (BVDV) is responsible for a wide spectrum of clinical disease that affects the respiratory, reproductive, immune and enteric systems of cattle [1]. Clinically BVDV is very diverse, causing acute disease ranging from mild or inapparent to severe, in addition to contributing to serious mixed infections as a result of BVDV-induced immunosuppression [2]. Furthermore, infection of the bovine foetus may result in abortion, teratogenic effects or the birth of persistently infected immunotolerant calves which act as a source of infection for susceptible animals [2]. BVDV is classified within the pestivirus genus of the Flaviviridae [3]. Other notable veterinary pathogens of the pestivirus genus include classical swine fever virus (CSFV) and border disease virus (BDV). These pestiviruses also show significant similarity to human hepatitis C virus in terms of genome structure and replication strategies. BVDV comprises a single-strand positive sense RNA molecule of approximately 12.5 kb which has a single open reading frame encoding a number of structural and nonstructural proteins which are cleaved from polyprotein precursors [4]. The structural proteins are the nucleocapsid and the three envelope glycoproteins E^{RNS}, E1 and E2 [5].

Currently modified live (MLV) and inactivated vaccines are used to protect cattle from BVDV infection, however none allow for differentiation between vaccinated cattle and those exposed to the virus via natural infection. The development of an effective subunit vaccine would allow such discrimination and enhance current efforts to control BVDV. To this end, evaluation of the host's immune responses to individual viral antigens has been the focus of much research over the last few years. The 53 kDa major envelope glycoprotein, E2, is the most immunogenic protein of BVDV and induces high titres of protective neutralizing antibodies after infection [6]. As such, the E2 protein of BVDV is a prime candidate for a subunit vaccine. E2 has been delivered as an antigen by many methods including recombinant protein [7,8], as a constituent of recombinant adenovirus [9], as a DNA vaccine construct [10–13] and in a DNA–protein prime-boost regime [14] with varying degrees of efficacy.

In this current study we chose to assess the level of protection obtained from using recombinant E2 protein as a vaccine. Subunit vaccines often depend upon a single protein to generate an effective immune response within the vaccinated host; as such both the quantity and antigenic authenticity of the recombinant protein can be crucial. Therefore, the choice of protein expression system is often influenced by the type of protein to be expressed, the degree of functional 'authenticity' required, the convenience of the system and yield of product.

The baculovirus insect cell expression system is one of the most widely used tools for the high level expression of heterologous proteins in a eukaryotic host [15–17]. Advantages include the high level

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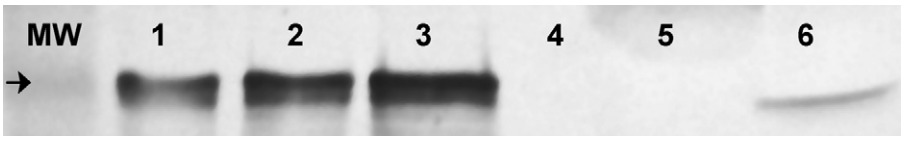

Fig. 1. A western blot image of E2 protein produced from either the baculovirus (brE2) or mammalian (mrE2) expression system. (Mw) molecular weight marker, arrow depicts 53 kDa; lanes 1–3 brE2 (50, 100 and 150 ng, respectively); lane 4 uninfected Sf9 cells; lane 5 untransfected MDBK cells; lane 6 mrE2 (17 ng).

of foreign protein produced as well as the insect cells being capable of post-translational modifications similar to those of mammalian cells. In spite of these advantages there is a fundamental difference in the protein glycosylation pathways of insect and mammalian cells; whilst mammalian cells produce complex *N*-glycans with terminal sialic acids, insect cells mainly produce simpler *N*-glycans comprising terminal mannose residues [16,18–20]. N-linked glycosylation present on most proteins on the surface of enveloped viruses can play a major role in folding, stability and antigenicity as well as the biological functions of these proteins [21,18].

There are a wide variety of expression vectors and mammalian cell lines to support recombinant protein production. Mammalian cell-based expression systems typically yield a more authentically processed active protein and therefore are ideal for the production of native protein that will be used in functional studies or applications. However, a disadvantage of mammalian expression systems can be the comparatively small amount of recombinant protein produced.

In this study we sought to evaluate and compare the protective efficacy of E2 glycoprotein of BVDV expressed in mammalian and baculovirus systems. The mammalian system chosen had been previously used for the expression of glycoprotein gD from Bovine Herpesvirus in bovine cells [22]. The baculovirus system used was commercially available. Recombinant mammalian E2 (mrE2) and baculovirus E2 (brE2) proteins were formulated with Quil A adjuvant and used to vaccinate cattle. Two trials were undertaken. In experiment 1, a mrE2 vaccine containing the maximum payload that was practically possible was compared with a brE2 vaccine with an optimized payload (dose titration of BrE2 performed previously and not described here). The level of protection against BVDV challenge afforded to cattle post-vaccination was assessed by comparing clinical, haematological, serological and virological responses of vaccinated calves and unvaccinated controls. A second study, experiment 2, was subsequently undertaken to assess the immune response to an equivalent specific dose of each antigen.

2. Materials and methods

2.1. Generation of recombinant proteins

The generation of a recombinant baculovirus expressing E2 from the non-cytopathic BVDV type 1 strain Ky1203nc has been described previously [11]. Briefly C-terminally truncated E2 cDNA from Ky1203nc derived from RT-PCR of infected foetal bovine lung (FBL) cells was cloned into the baculovirus vector pMelBac (Invitrogen). This vector was subsequently used to produce a recombinant baculovirus designated AcE2. All vector constructs were sequenced to confirm their integrity prior to generating recombinant baculoviruses via homologous recombination with Bac-N-Blue viral DNA (Invitrogen). AcE2 was amplified to give high titre stocks. Infection of serum-free adapted Sf9 insect cells with AcE2 led to the expression of recombinant E2 protein. Typically, Sf9 cells were infected with AcE2 at an moi of 10 and incubated at 28 °C for 96 h. The cells were pelleted (1000 × *g*, 10 min) and lysed in 0.1 M Tris pH7.4 containing 1% Triton-X-100 at 4 °C. The cellular debris was removed by centrifugation and the lysate stored at −70 °C. The amount of E2 in each preparation was estimated by densitometric analysis of western blots using Imagemaster ID Elite software version 4.10. Essentially the same C-terminally truncated E2 sequence from Ky1203nc was cloned into the mammalian expression vector pBAM, under the control of the bovine homologue of the human Hsp70a gene promoter [22]. The Ig κ-chain leader sequence was also introduced into the vector, upstream of the E2 coding sequence. The plasmid was transfected into MDBK cells and stable cell lines generated via G418 selection. Drug resistant clones were screened and those clones expressing the highest levels of E2 protein identified. Expression of recombinant E2 was induced and cells harvested at 6 h post-induction. The cells were then processed as described above. Protein concentration of E2 was assessed via densitometry in a western blot against a purified recombinant brE2 standard of known concentration. A representative western blot depicting both antigens is shown in Fig. 1.

2.2. Animals and experimental design 1

Fifteen Holstein-Friesian calves were obtained from a BVDV free supplier and tested before inclusion in the study to ensure they were BVDV antibody and antigen free. Five calves were allocated to each of three treatment groups according to a random block design based on age. After an appropriate acclimatisation period, each candidate vaccine was administered subcutaneously, three times at intervals of 21 days (study days −63, −42 and −21). The first group were vaccinated with doses of 100 μg of baculovirus-expressed E2 protein (brE2); second group with doses of 5 μg of mammalian expressed E2 protein (mrE2) and the third group consisted of the unvaccinated controls. On each occasion blood samples for serology were taken.

2.3. Challenge and sampling phase

The challenge BVD virus (an early passage, genotype 1 non-cytopathogenic field isolate) was amplified and subsequently backtitrated in FBL cells. The original isolate, denoted #456497, was kindly donated by Dr. Trevor Drew, VLA Weybridge, UK. Pre-challenge baseline measurements of haematological parameters and rectal temperatures were taken on study day −2, −1 and 0. On day 0 all animals were challenged intranasally with 2×10^6 TCID of virus #456497. Following challenge, rectal temperatures were taken daily, until day 10, and EDTA blood samples for haematology until day 14. EDTA blood samples were processed using an automated haematological analyser (Cell Dyn, Abbot) to determine total leucocyte counts. A further EDTA blood sample was taken for virus isolation from buffy coat on days 0, 3, 5, 6, 7, 10, 14 and 21. In addition plain blood samples were taken for serology on days 0, 7, 14 and 21. Nasopharyngeal swabs were taken on days 0, 3, 5, 6, 7, 10 and 14.

2.4. Animals and experimental design 2

Due to the different quantity of E2 antigen present in each vaccine of experiment 1, a second study was undertaken using comparable doses of E2 antigen. BVDV free cattle were obtained and the 10 calves were placed into two equal treatment groups. Test substances were administered three times, at intervals of 21 days via

the subcutaneous route. One group were vaccinated with 5 μg of baculovirus expressed E2 (brE2) and the second group were vaccinated with 5 μg mammalian expressed E2 (mrE2). These calves were not challenged but samples were taken for serology on days 0 (V1); day 21 (V2); day 42 (V3); day 63 (3 weeks post-final vaccination).

2.5. Serology

Detection of antibodies to BVDV in serum was performed using an indirect ELISA [23]. The antigen used in the ELISA was the cytopathic reference strain NADL. The results are expressed in ELISA units/ml, with the value of a single point dilution interpolated from a reference serum standard curve. Values of ≤100 units/ml are considered negative. The E2 specific ELISA, using purified, antigen was performed as described previously [11]. Levels of neutralizing antibody were established as described before [24]. The results are expressed as the reciprocal of the serum dilution at which 50% of the virus was neutralized.

2.6. Virus isolation from buffy coat samples and nasopharyngeal swabs

All EDTA blood samples taken for virology from day 0 onwards were prepared by extracting and washing the total white blood cell pellet. The presence of virus was then determined in these samples after dilution. Briefly samples were inoculated in duplicate onto freshly prepared FBL cells in 24-well plates and incubated for 5 days. After freeze thaw and dilution of material the inoculum was passed once more onto cells seeded on sterile glass coverslips and incubated for a further 5 days. Presence of virus was detected via immuno-fluorescence staining using a polyclonal bovine anti-BVDV serum and a Cy-3-conjugated anti-bovine antibody (Stratech). Scoring was performed using a fluorescence microscope and appropriate filter sets. To detect BVD virus in nasopharyngeal swabs, the samples were diluted (1:5) in maintenance medium (Minimum Essential Medium containing 2% foetal calf serum and antibiotic/antimycotic solution) and then inoculated, in duplicate, onto freshly prepared FBL cells in 24-well plates and incubated for 5 days at 37 °C. After one round of freeze–thaw and dilution of material, the inoculum was passed once more onto fresh FBLs and incubated for a further 5 days. Presence of virus was detected via immuno-fluorescence staining as described above.

2.7. Statistical analysis

Statistical significance was evaluated using a One-way ANOVA test with post hoc Bonferonni adjustment for experiment 1, and Students *T*-tests for experiment 2 (SPSS version 15.0). Data that was not normally distributed was subjected to Log_{10} transformation and normality confirmed before statistical analysis was carried out.

3. Results

3.1. Experiment I—clinical and haematological observations

Mean rectal temperatures pre-challenge were similar for all groups (Fig. 2a). For the first few days post-challenge temperatures remained stable; however, there was a marked increase in rectal temperatures on day 7 post-challenge for animals within the unvaccinated control group. The control group mean temperature peaked at 40.8 ± 0.3 °C. This contrasted with the temperatures obtained from animals of the vaccinated groups which remained within the normal range throughout. Statistical analysis confirmed that mean temperatures recorded for the control group were significantly higher than for the group vaccinated with brE2 ($p = 0.021$) and mrE2 vaccinates ($p = 0.023$) on day 7. This demonstrated that both recombinant vaccines were successful in preventing pyrexia in these animals.

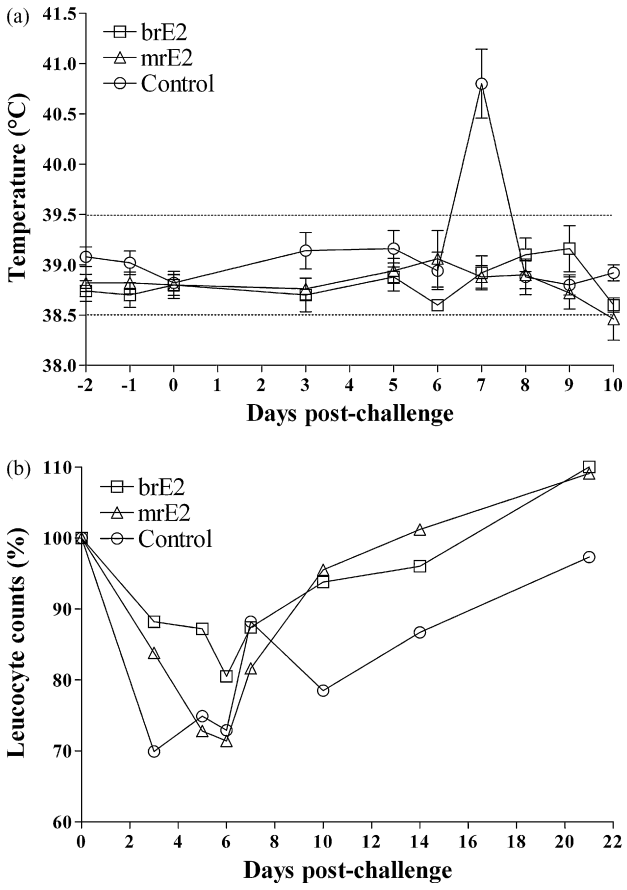

Fig. 2. Temperature and leucocyte fluctuations following BVDV challenge of calves vaccinated with brE2 (□), mrE2 (△) or unvaccinated (○) (experiment I). (a) Mean rectal temperatures ±S.E.M. of each vaccine group; dotted line indicates normal range. (b) Mean total leucocyte counts expressed as a percentage of pre-challenge values.

To determine the extent of leucopaenia, counts were made from blood samples for three consecutive days prior to challenge. These values were averaged and each subsequent count was described as a percentage of the pre-challenge baseline values (Fig. 2b). Pre-challenge, the mean leucocyte counts for each group remained similar and within the normal range ($4–12 \times 10^6$/ml) (data not shown). There was a characteristic leucopaenia at 3 days post-challenge in the control group. The control group exhibited a 31% reduction against pre-challenge baseline leucocyte counts compared with only a 12% reduction in group brE2 or a 16% reduction in mrE2 vaccinates, although a reduction in leucocyte counts was seen on days 5 and 6 post-challenge in the mrE2 vaccinated group (reduction of 27% and 29%, respectively) and on day 6 post-challenge for the brE2 vaccinated group. However, by day 10 post-challenge vaccinated animals had recovered to pre-challenge leucocyte values which contrasted with the control group counts which were still 22% below mean pre-challenge levels at the same time point.

3.2. Experiment 1—serology

All calves of the control group were seronegative at the time of challenge and remained so until at least 14 days post-challenge (Fig. 3). Early signs of a specific immune response were observed in vaccinated groups pre-challenge. Three calves from the brE2 group

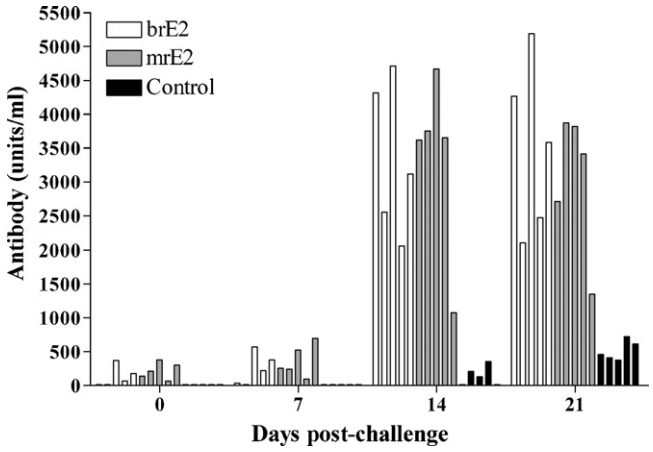

Fig. 3. Serum antibody responses in calves vaccinated three times with brE2 (□), mr E2 (▒) or unvaccinated (■) and subsequently challenged with BVDV (experiment I). Data shows anti-BVDV antibody titres recorded for each individual in each group post-challenge (day 0).

and four from the mrE2 group had seroconverted, in an indirect ELISA, following vaccination and were antibody positive at challenge. Post-challenge, the antibody titres continued to rise in the vaccinated groups. Neutralising antibodies were assessed against the challenge strain of virus rather than the homologous vaccine strain. On the day of challenge animals vaccinated with either brE2 or mrE2 possessed measurable neutralizing antibody titres (Table 1). In contrast, control group neutralizing antibodies were not detected until 14 days post-challenge.

3.3. Experiment 1—virology

Virus isolation from buffy coat samples was undertaken (Table 2). All calves were negative for virus on the day of challenge. In the control group, infectious virus was first isolated 5 days post-challenge and this individual remained viraemic until at least day 7. Virus was isolated from buffy coat samples from four out of five control calves. All calves had cleared virus by 10 days post-challenge. Virus was first recovered from vaccinated groups at 6 days post-challenge, from one individual in each group. Similar results were seen in both vaccinated groups with infectious virus being isolated at day 7 post-challenge from four out of five animals.

The results of virus isolation from nasopharyngeal swabs showed that infectious virus was first detected in the control group 3 days post-challenge (Table 3). Virus was isolated from all individuals of the control group; from two calves at four time points; two calves at three time points and at one time point from the fifth calf. In contrast virus isolation from nasal swabs taken from vaccinated animals was more infrequent. In the brE2 vaccinated group, two individuals showed no evidence of nasal shedding throughout the duration of the sampling regime and infectious virus was isolated from three individual calves on only one study day. Only one individual of mrE2 group showed any evidence of nasal shedding, virus could not be detected in the nasal mucosa at any time point from the remaining 4 calves in this group.

Table 1
Neutralising antibody titres following vaccination and subsequent challenge (day 0).

Group	Calf ID	Seroneutralisation titres	
		Day 0	Day 14
brE2	2	226	6400
	4	113	1600
	19	160	1128
	23	452	6400
	18	452	2255
mrE2	24	400	6400
	14	159	1600
	22	113	1128
	7	159	6400
	16	113	2255
Control	20	<10	564
	10	<10	<100
	1	<10	<100
	3	<10	<100
	15	<10	282

Table 2
Virus isolation from buffy coat following BVDV challenge.

Group	Calf ID	Study day post-challenge						
		0	3	5	6	7	10	14
brE2	2	–	–	–	+	+	–	–
	4	–	–	–	–	–	–	–
	19	–	–	–	–	+	–	–
	23	–	–	–	–	+	–	–
	18	–	–	–	–	+	–	–
mrE2	11	–	–	–	–	+	–	–
	17	–	–	–	–	–	–	–
	12	–	–	–	–	+	–	–
	6	–	–	–	–	+	–	–
	25	–	–	–	+	+	–	–
Control	20	–	–	–	–	–	–	–
	10	–	–	+	+	+	–	–
	1	–	–	–	+	+	–	–
	3	–	–	–	+	–	–	–
	15	–	–	–	+	–	–	–

Following vaccination and challenge, samples were taken for 14 days. Virus isolation from buffy coats was performed by immunofluorescent assay after two passages on FBL cells.

3.4. Experiment 2—serology

Due to the disparity in E2 protein content of each vaccine, primarily due to limitation of protein production when using a mammalian expression system, we chose to undertake a second experiment in which the dose of E2 in each vaccine was directly comparable. In this experiment all calves were vaccinated with three doses at 5 μg E2/dose of either vaccine. The results of the BVDV antibody ELISA (Fig. 4a) showed that calves vaccinated with

Table 3
Virus isolation from nasopharyngeal swabs.

Group	Calf ID	Day post-challenge						
		0	3	5	6	7	10	14
brE2	2	–	–	–	–	–	–	–
	4	–	–	–	+	–	–	–
	19	–	–	–	+	–	–	–
	23	–	–	–	+	–	–	–
	18	–	–	–	–	–	–	–
mrE2	11	–	–	–	–	–	–	–
	17	–	–	–	–	–	–	–
	12	–	–	–	+	–	–	–
	6	–	–	–	–	–	–	–
	25	–	–	–	–	–	–	–
Control	20	–	–	–	+	+	+	–
	10	–	+	+	+	–	–	–
	1	–	+	+	+	+	–	–
	3	–	–	–	+	–	–	–
	15	–	+	+	+	+	–	–

Following vaccination and challenge with BVDV, nasopharyngeal swab samples were taken. Virus was detected by immunofluorescent assay, using polyclonal BVDV serum and Cy3-labelled anti-bovine secondary antibodies, after passage of samples on FBL cells. (+) denotes virus isolated from samples.

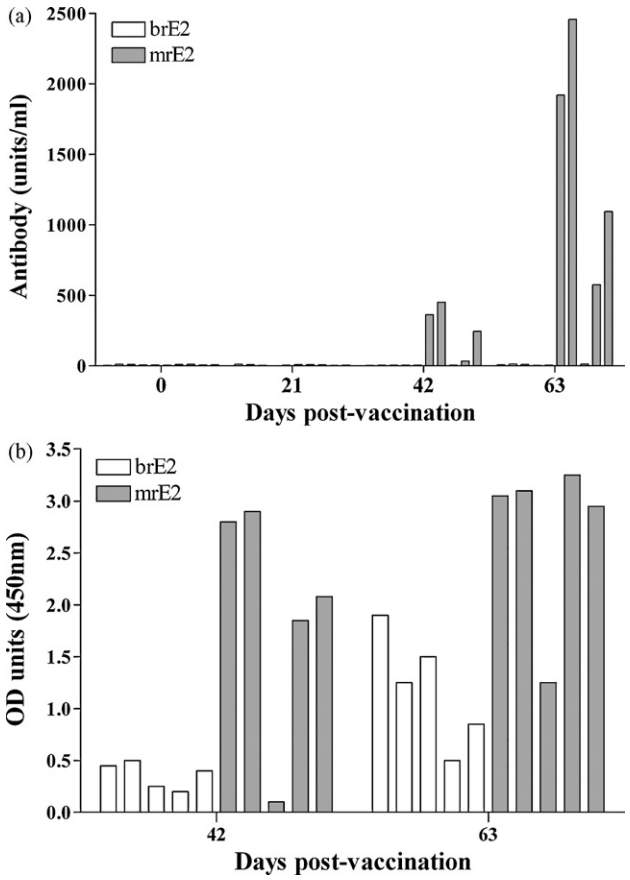

Fig. 4. Serum antibody responses in calves vaccinated three times with 5 μg brE2 (□) or mrE2 (■) (experiment II). (a) Anti-BVDV antibody titres recorded for each individual in each group. (b) E2-specific antibody responses.

only 5 μg of baculovirus expressed E2 failed to produce an antibody response to BVDV which contrasted with calves vaccinated with an equivalent dose of mammalian expressed E2. Interestingly, three out of five calves vaccinated with mrE2, had seroconverted after two vaccinations with a third calf seroconverting by 3 weeks post-final vaccination. An E2 specific antibody ELISA was carried out on samples from study days 42 (V3) and 63 (Fig. 4b). On study day 42 four out of five mrE2 vaccinates had high anti-E2 antibody levels and by day 63 all members of this group were displaying high levels of antibody. Calves vaccinated with brE2 had far lower levels of E2 specific antibodies than those vaccinated with mrE2, however there was a noticeable increase in the antibody level in this group on day 63.

4. Discussion

DIVA (differentiating infected and vaccinated animals) or marker vaccines have been the subject of much discussion and research over the last few years. Initially some vaccines contained an additional component to allow identification of vaccinates although it is now widely accepted that vaccines which lack some of the antigenic components of the infectious agent are often more useful constructs for use in the field. The ultimate marker vaccine would contain a single antigenic component of the infectious agent which was both highly antigenic, providing complete protection against infection, and conserved, so as to provide cross protection against different strains. For BVDV, research has been primarily focused on the highly antigenic E2 protein which does induce protection though with some variability between field strains [7–10,12–14].

In the current studies, we have evaluated the efficacy to afford protection against BVDV challenge, of two different preparations of recombinant BVDV E2 antigen—E2 expressed from either a baculovirus (vaccine candidate brE2) or a mammalian expression system (vaccine candidate mrE2). Broadly speaking the two systems offer different options in the choice of quality and quantity of the protein antigen being expressed. Baculovirus expression systems generally offer the ability to produce relatively large quantities of foreign protein with protein processing pathways similar to mammalian cells producing some level of antigenic authenticity. Mammalian cell expression systems produce more authentic protein folding, complex glycosylation and a broad range of post-translational modifications, although they are generally more limited in the quantity of antigen that can be produced, particularly in a laboratory-scale system.

In experiment 1 of this study, the results suggest that both vaccines were effective in preventing pyrexia and at reducing the duration of the leucopenia observed in response to BVDV infection. A significant rise in rectal temperatures was seen in the unvaccinated control group on day 7 post-challenge, typical of BVDV infections. In contrast rectal temperatures recorded from calves vaccinated with either candidate brE2 or mrE2 remained within the normal range throughout. Some protection against transient leucopaenia was also evident and by day 10 post-challenge vaccinated animals had recovered to pre-challenge leucocyte values which contrasted with the control group.

Analysis of viraemia in control and vaccinated animals gave a more complex picture. While neither vaccine induced complete protection, all vaccinated animals showed an encouraging degree of reduction of virus shedding from the nasal mucosa. Two calves from group brE2 and four calves from group mrE2 shed no detectable virus at all while virus could be isolated from all individuals of the unvaccinated control group, with four calves shedding infectious virus for a minimum of 3 days. The results of virus isolation from buffy coat demonstrated that infectious virus could be isolated from 4 out of 5 individuals in each group, vaccinated or controls, suggesting that neither vaccine candidate had been successful in completely preventing viraemia. The study was high stringency using antigenically heterologous vaccine and challenge strains.

Antibody responses to BVDV challenge were assessed by BVDV antibody ELISA and virus neutralisation tests. The level of neutralizing antibody generated by vaccination is important for evaluating candidate vaccines as high titres of passively derived antibody confer protective immunity against BVDV [25,26]. Both vaccine candidates would make DIVA vaccines and we demonstrated that all animals in the study were seronegative for BVDV including E2 and the nonstructural protein NS3 prior to recruitment. All calves of the control group were seronegative to BVDV at the time of challenge and at 2 weeks post-challenge low levels of BVDV neutralizing antibody and anti-NS3 antibodies could also be detected in some individuals. In contrast, several vaccinated animals had seroconverted specifically to E2 following vaccination (three calves of group brE2, and four calves of group mrE2) and were E2 antibody positive at challenge. Post-challenge, the mean levels of antibody for all vaccinated animals increased rapidly.

In this first study, while both E2 vaccine preparations gave partial protection in the face of BVDV challenge, the dose of E2 protein delivered by each expression system varied, being 100 μg/dose in the baculovirus produced vaccine compared with only 5 μg/dose for the mammalian expressed E2. In our hands it has proved unrealistic to achieve a higher level of recombinant E2 expression from this mammalian system. Kowalski and co-workers produced approximately 500 μg recombinant bovine herpesvirus glycoprotein gD from 4×10^7 cells by repeat induction using this mammalian expression system [22], therefore it is conceivable that further screening for a higher expressing clone and repeated induction

may lead to an increase in yield of recombinant E2. There is the added possibility that the utilisation of a cryptic splicing signal in the BVDV E2 mRNA sequence could have reduced the amount of authentic protein being expressed as observed for the E2 of the related CSFV [27]. The presence of RNA splicing signals in the cDNA construct of the E2 gene of classical swine fever virus affected its expression. If proven, this loss could be reduced by using a synthetic gene construct for expression of E2 as was undertaken in a recent study which used the same mammalian expression system to produce recombinant protein from a codon optimised E2 construct. After monoclonal antibody affinity purification and concentration of E2 this afforded an antigenic dose of 35 μg [14], although this is unlikely to be an economically viable approach for production of a commercial veterinary vaccine.

Although high recombinant protein yield is a fundamental challenge in mammalian protein expression systems, it has been previously demonstrated that the level of functional expression from mammalian cells can be higher than from the baculovirus expression system [28]. In an experiment which compared a variety of expression systems Tate and colleagues determined that a significant proportion of the recombinant serotonin transporter (SERT) protein expressed from the baculovirus system was functionally inactive. This highlights the potential pitfalls of a simple comparison of quantity of protein expressed. In experiment 1, a similar level of protection was achieved with two very different E2 antigen doses, therefore we hypothesise that either the level of functional, antigenically authentic protein from the mammalian system was higher or that it should be possible to reduce the protein dose of the baculovirus expressed E2 vaccine further. In experiment 2 the amount of baculovirus expressed E2 used for immunisation was 5 μg/dose, the same as that for the mammalian expressed product. The results demonstrate that this significantly reduced the level of antibody production in those vaccinates administered the lower dose of baculovirus expressed E2 antigen.

Both our recombinant E2 protein preparations bind polyclonal and monoclonal antibodies raised against E2 in western blots and ELISA and when delivered as vaccines provide some degree of protection to live virus challenge. Tunicamycin studies demonstrated glycosylation of E2 had occurred (data not shown) although the nature of the carbohydrate moieties added were not determined. Experiments removing putative N-linked glycosylation sites on BVDV E2 expressed in baculovirus have shown their importance for the functional activity of the E2 protein *in vitro* [29]. Studies by Tate and colleagues demonstrated that a major proportion of heterologous protein expressed via the baculovirus system was misfolded [28], and it can be assumed that this would reduce its ability to induce protective immunity including antibodies. Our own results demonstrating comparable efficacy with delivery of only 5% of the protein payload from mammalian cells would also suggest that these factors may be influencing functional activity of the brE2 protein antigen. Additionally, insect cell carbohydrate moieties comprising mainly high mannose-type core structures [30,31] may influence antigenicity and biological activity of the protein. Furthermore, the absence of terminal sialic acids on recombinant glycoproteins can lead to a short half-life of the protein in vivo [32]. The results obtained from the second cattle study would indicate that the antigenic authenticity of the mammalian expressed E2 is greater than that of the baculovirus expressed E2.

In conclusion both brE2 and mrE2 antigens provided partial protection to cattle from BVDV challenge, as evidenced by humoral responses, lack of pyrexia and a reduction in both leucopaenia and nasal shedding. At the higher dose (100 μg) the protection induced by the baculovirus expressed antigen was comparable to the mammalian expressed protein. However, this level of protection diminished when the baculovirus expressed antigen dose was reduced (5 μg), demonstrating the impact that both the type of expression system used and the antigenic dose have on protection. The production of a commercial vaccine product based on recombinant protein must evaluate the cost benefit analysis of function versus yield in this light.

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