POTENTIAL OF MODIFIED VACCINIA ANKARA (MVA) AS A VACCINE DELIVERY VECTOR FOR FOOT-AND-MOUTH DISEASE VIRUS (FMDV).

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ABSTRACT

Although FMDV inactivated vaccines are currently used with success there are a number of modifications that could improve their efficacy. These improvements aim at increasing the duration of immunity, stimulating strong cellular immune responses and/or providing differential diagnostic capability, and can be achieved by the use of live viral vectors, such as MVA, expressing selected FMDV antigens.

Our aim is to elucidate whether MVA viruses expressing FMDV proteins could be used, either on their own or in combination with conventional vaccination, to improve the immunity against the disease.

The P1 and the 3C/D regions of the A22-Iraq64 FMDV strain were cloned in the standard vaccinia transfer vector pSC11 downstream of the vaccinia early / late P7.5 promoter to generate MVA-P1 and MVA-3CD viruses. Expression of the FMDV expression cassettes was characterised by RT-PCR, immunofluorescence and Western blotting prior to vaccination of cattle with the recombinant viruses. The recombinant MVA-P1 were generated and grown to high titres in chicken fibroblasts (CF). Attempts to recover a recombinant MVA-3CD were unsuccessful up to date. Transcription of the FMDV genes from recombinant MVA-P1 infected cells was confirmed by RT-PCR amplification of P1 sequences from extracted RNA. Levels of expression vary considerably between cell lines, as revealed by immunofluorescence. Thus, P1 expression was detected in CF, LB-9 (bovine skin dermis cells) and P815 (mouse mastocytoma cell line) up to multiplicities of infection of 0.01, 1 and 10 respectively. Expression of P1 could not be detected in BK (bovine kidney cells). A protein band of approximately 80 KDa, corresponding to P1, could be detected with FMDV A22-specific rabbit polyclonal antiserum in immunoblots of MVA-P1 infected CF lysates. Furthermore, when MVA-P1 infected cells were co-transfected with a pSC11 plasmid encoding 3CD downstream of the vaccinia P7.5 promoter evidence of P1 processing was obtained.

We conclude that recombinant MVA-P1 can be easily generated and grown to high titres and that it expresses the FMDV P1 antigen in bovine cells in vitro showing therefore its potential to be used in FMD vaccination. Further work is currently underway to test its efficacy as a vaccine in vivo.

1. INTRODUCTION

Foot-and-mouth disease is a highly infectious disease of cloven-hoofed animals caused by (FMDV) which belongs to the Aphthovirus genus of the family Picornaviridae. The disease causes vesicular lesions in the tongue and oral mucosa, feet, snout and teats resulting in high morbidity and low mortality. However, in young animals the infection can be fatal due to myocarditis. The economic impact of FMD is huge due to export loses and sharp decrease in productivity of the affected farms (Grubman and Baxt, 2004).

Control of FMD is achieved by slaughtering infected and in-contact animals and restricting movement of infected products or by means of emergency vaccination in free countries or mass vaccination in endemic countries. However, vaccination is not always implemented, especially in disease-free countries, due the costs associated with the ban on exports from these countries until the disease-free status is re-gained. This happens only after 6 months from the last case of FMDV if vaccination is employed as opposed to only 3 months if slaughter without vaccination is implemented (Kitchin et al., 2007).
All currently available FMDV vaccines are based on cell culture derived preparations of whole virus, chemically inactivated and blended with oil or aluminium-saponin adjuvant to potentiate the immune response to vaccination. Killed vaccines are unstable at ambient temperature, and after formulation must be kept cool until administered. Since they are produced from live FMDV, strict biocontainment is necessary to exclude the risk of spreading virus from manufacturing plants (Grubman and Baxt, 2004; Kitchin et al., 2007; Doel, 2003). An animal immunised against one strain of FMDV may still be susceptible to another. Therefore, a number of vaccine strains for each serotype, particularly A and O, are required to cover the antigenic diversity and it is essential to monitor for the appearance of new strains internationally (one of the prime functions of the World Reference Laboratory). Depending on the dose and on the severity of challenge, FMDV vaccines may protect against disease within 4 to 5 days of vaccination and such protection may endure for approximately 6 months (Barnett et al., 2002).

While existing vaccines have been associated with notable success, there are a number of areas where improvements would dramatically enhance the prospects for control of a FMD outbreak in a previously free country.

1) More rapid onset of protection.
2) Wider spectrum of protection against different strains.
3) More potent immunity preventing virus replication and development of viral carriers.
4) Better discrimination of vaccinated animals that go on to become infected.
5) More thermostable.
6) Easier and safer to make.
7) Easier to administer.

Currently we are attempting to improve the efficacy of killed vaccines by co-administering them with recombinant modified Vaccinia Ankara (MVA) viruses expressing FMDV antigens, in particular P1 (precursor of capsid proteins) and 3C/D (encoding the viral protease and RNA polymerase respectively). This may provide more T-cell mediated antibody responses as current vaccine is chemically inactivated and devoid of non-structural component. Recombinant MVA has been utilised successfully as a viral vaccine vector for many diseases being especially effective at inducing cellular immune responses when administered in vaccination regimes in combination with other antigen delivery systems (i.e. DNA, sub-unit vaccines) (Gilbert et al., 2006).

2. METHODS

2.1. Cells, viruses and plasmids
Primary chicken embryo fibroblasts (CEF) from gnotobiotic chickens were obtained from the Microbiological Services, Institute for Animal Health (IAH), Compton. The avian cell line DF-1, primary bovine thyroid cells (BTY), bovine dermal cells (LB-9) and bovine kidney (BK) were obtained from the Central Services Unit, (IAH), Pirbright. The P815-BLA cell line, a mouse mastocytoma cell line stably expressing bovine MHC-I, was obtained from Dr Shirley Ellis, IAH, Compton. All cell lines were grown in Dulbecco Minimal Essential Medium supplemented with Heps, penicillin-streptomycin, L-glutamine and foetal calf serum and propagated using standard cell culture techniques.

The standard vaccinia shuttle vector used was pSC11 (Chakhrabarti et al., 1985). Modified Vaccinia Ankara (MVA) was obtained from Dr Barbara Blacklaws (University of Cambridge).

2.2. Cloning and generation of recombinant MVA
The cDNA of the complete P1 and 3CD regions of FMDV were derived from total RNA (extracted with Trizol Reagent) from BTY cells infected with the FMDV A22 Iraq 64 strain. A one-step RT-PCR reaction (SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity, Invitrogen) using FMDV specific primers bearing SmaI restriction sites and start and stop codons was used. After digesting the P1 and 3CD DNA amplicons with SmaI, the DNA fragments were inserted in the SmaI site of pSC11, downstream of the P7.5 vaccinia early / late promoter.

Purified plasmids were subsequently used to transfect MVA infected CEF and / or DF-1 cells. Briefly, 24h-old cell monolayers were washed with Hank’s balanced salt solution (HBSS) before infection with MVA using a multiplicity of infection of 1. Two hours later the cells were washed again and transfected using the recombinant pSC11-P1 or pSC11-3C/D plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer recommendations. The cells were scrapped when CPE reached 80% of the cell sheet and the cells were harvested, centrifuged at low speed and resuspended in HBSS. The harvested cells were stored at -80°C until used in plaque assays.
2.3. Plaque assays
Nearly confluent cell monolayers were inoculated with serial dilutions of the transfection harvest and after 2 hours at 37°C and removing the inoculum the cells were overlayed with 10% DMEM containing 1% agarose. The cells were incubated at 37°C and a second agarose overlay containing 400 ug/ml of X-gal was added when cells began to show CPE (usually 48-72 hours). Blue plaques produced by recombinant MVA were picked and subjected to various rounds of plaque purification until a pure stock was obtained. This was grown to high titres in DF-1 cells and used to characterise the expression of the inserted FMDV sequences.

2.4. Immunofluorescence
Cells grown in glass cover slips were fixed with PBS containing 4% formaldehyde and 0.4% Triton-X-100, washed with PBS and blocked with PBS containing 1% bovine serum albumin. The primary antibody used was a rabbit polyclonal antiserum raised against inactivated FMDV A22 (a gift from Nigel Ferris, IAH, Pirbright) diluted in blocking buffer. After washing with PBS the cells were incubated with FITC-conjugated goat antibody to rabbit IgG (Zymed) diluted 1/200 in blocking buffer. After a final wash with PBS and then water the cells were mounted in Vecta-shield mounting medium with DAPI and observed on a UV light microscope.

2.4. Western blot
Cells were lysed in lysis buffer [(20mM Tris-HCl, 150mM NaCl, 1% sodium deoxycholate, 1% Tergitol, 0.1%, SDS, 2mM EDTA, supplemented with protease inhibitors (all supplied by Sigma-Aldrich)] and diluted 1:1 in 2x protein loading buffer (National Diagnostics) before heating at 95°C for 5 minutes. Samples were run on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane using the iBlot Dry blotting device and iBlot membranes (Invitrogen). The FMDV A22 specific rabbit antiserum was used as the primary antibody. The Western Breeze Chromogenic kit-Anti Rabbit was used for the rest of the procedure according to the manufacturer recommendations.

3. RESULTS
A recombinant MVA virus encoding the P1 segment of FMDV A22 strain was generated and grown to high titres following the procedures indicated in the materials and methods section. The expression of the P1 protein in various cell lines was evaluated and characterised.

3.1. Detection of P1 sequences by RT-PCR from MVA-P1 infected CF cells
In order to determine whether the P1 expression cassette was functional we performed an experiment aimed at detecting P1 sequences by RT-PCR from extracted RNA samples of MVA-P1 infected cells using P1 specific primers (forward primer: 5’-tacatggtggcgtacgtt-3’; reverse primer: 5’-ccgtagttttcaacagtggt-3’). To maximise the chances of detection of P1 RNA we infected CF cells with MVA-P1 at an m.o.i. of 1 and extracted the RNA after 24 hours of infection (Fig. 1). Sensitive X-gal staining of fixed MVA-P1 infected CF cells at 24 hours post-infection revealed that the reporter Lac-Z gene was expressed in 100% of the cells indicating that P1 is also probably expressed abundantly. Indeed, P1 amplicons were easily detected from the RNA extracts of MVA-P1 infected CF cells. The detection of P1 amplicons from the DNA-se treated samples confirmed the P1 expression was not due to contaminating MVA-P1 viral DNA.

3.2. Detection of P1 expression by indirect immunofluorescence
Immunofluorescence experiments on fixed MVA-P1 infected cells were performed to determine the levels of protein expression in different cell lines, including cells from bovine origin. Detection of P1 was detected with very high sensitivity in CF (at 24 and at 48 hours) up to multiplicities of infection of 0.001 (Fig 2). Expectedly, expression levels were reduced in bovine dermal cells (LB-9) and bovine dendritic cells (up to an m.o.i. of 10) in comparison to those achieved in CF. Expression of P1 in bovine kidney cells could not be detected. We also performed experiments in P815 cells that have been stably transfected with bovine MHC-I. This cell line will be a very useful tool to examine the cytotoxic cell responses of FMD infection in cattle. Expression of P1 was achieved in this cell line up to m.o.i. of 0.1 (Fig. 3).

3.3. Western blot analysis of MVA-P1 infected cells
The analysis of P1 expression in MVA-P1 infected CF and BK cells (Fig 4) are consistent with the results obtained by immunofluorescence. Bands of approximately 76 kDa were obtained in MVA-P1 infected CF cell lysates prepared at 24 and 48 hours post-infection. This was not the case for the BK cells. MVA-3CD was not available at the time these experiments were performed but a vaccinia
transfer vector encoding the 3CD segment of FMDV A22 (pSC11-3CD) was made and used to transfect MVA-P1 infected CF cells to check whether the P1 protein could be cleaved by the expressed 3C. Western blot experiments using lysates of MVA-P1 infected and pSC11-3CD transfected cells revealed, apart from P1, extra bands of approximately 31 kDa and 20 kDa, corresponding to the sizes of VP0 and VP1 and VP3. These extra bands could not be observed in any other cell lysate samples (Fig 5).

4. DISCUSSION

Recombinant MVA viruses expressing antigens have been utilised profusely as a vaccine strategy to induce immunity against viral diseases. Specifically they have been particularly effective at inducing cell mediated immune responses in combination with other vaccine antigen delivery methods or formulations when administered as a boosting agent. It is our goal to administer MVA recombinant viruses expressing FMDV proteins to cattle on their own or in combination with inactivated FMDV vaccines to enhance the cellular component of the immune response and therefore increase the strength and duration of immunity to FMDV. The primary objective of our research is to provide additional T cell help stimulus to antibody formation during the induction phase of the immune response as well as inducing cytotoxic T cell responses. For this, we use the capsid precursor protein (P1) (capsid antigens are present in the conventional vaccine) and the 3CD proteins (known to hold epitopes for CTL) of FMDV. Therefore, the processing of the P1 into individual capsid protein components of the virion is not a requirement of our strategy since the B cell epitopes will be provided by the conventional FMDV vaccine.

We have generated a recombinant MVA virus expressing P1 using standard molecular biology techniques. The virus was grown to high titres in the DF-1 chicken fibroblast cell line. The expression of the foreign gene is driven from the constitutive vaccinia promoter P7.5 and the protein was detected by immunofluorescence in various cell lines and also by Western blot. The latter experiments confirmed the identity of the protein by its size and further processing when the protease 3C was co-expressed with P1 in MVA-P1 infected cells transfected with pSC11-3CD. This processing was not strictly necessary to test our hypothesis that MVA-P1 and or MVA-3CD can improve FMDV vaccine efficacy but it could represent a strategy to produce FMDV capsids in vitro. Further studies are currently being performed to characterise the expression and processing of P1 into capsid components using the techniques described in this paper.

We have shown that the expression of P1 was achieved in avian as well as bovine cell lines. However, the levels of expression were reduced in the latter, reflecting the known replication characteristics of the avian adapted MVA virus. Despite these differences, we do not anticipate a failure of MVA-P1 vaccinated cattle to mount a measurable immune response to FMDV since a recombinant MVA carrying African horse sickness (AHS) virus VP2 was capable of stimulating virus neutralising antibodies to AHS virus in ponies after vaccination despite expression levels of VP2 were reduced in equine dermal cells in comparison with avian fibroblasts (Castillo-Olivares J., unpublished observations).

5. CONCLUSIONS

- We have generated an MVA-P1 expressing the P1 antigen and that has the potential to induce FMDV specific immunity in cattle.
- Vaccination experiments using MVA-P1 in combination with conventional vaccine are currently being performed.

REFERENCES

Fig 1a)

Fig 1b)
Figure 2: Detection of P1 in MVA-P1 infected chicken embryo fibroblasts (a) and bovine skin fibroblasts (LB-9) (b) using various multiplicities of infection (indicated in each photo of each panel). Cells were fixed with 4% formaldehyde, 0.4% TX100 at 48 hours post-infection and probed with rabbit anti-FMDV A22 polyclonal antiserum. After incubation with the appropriate FITC-conjugate cells were mounted with DAPI mounting medium and observed under fluorescence microscope.
Fig. 4: Western blot of MVA-P1 (lanes 1, 3, 5, 7) and MVA (2, 4, 6, 8) infected cell lysates prepared at 24 (1, 2, 3, 4) and 48 (5, 6, 7, 8) hours post-infection. Proteins were probed with A22 FMDV specific rabbit polyclonal antiserum. The secondary antibody was an Alkaline Phosphatase conjugated anti-rabbit IgG. Results were revealed using a chromogenic substrate. The ‘Rainbow’ molecular weight marker was used.

Fig. 5: Western blot of MVA-P1, MVA and mock infected cell lysates prepared at 14 hours post-infection. Half of the cell samples were transfected with pSC-3CD two hours post-infection. Proteins were probed with A22 FMDV specific rabbit polyclonal antiserum. The secondary antibody was an Alkaline Phosphatase conjugated anti-rabbit IgG. Results were revealed using a chromogenic substrate. The ‘Rainbow’ molecular weight marker was used.