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Expression in cattle of epitopes of a heterologous virus using a recombinant rinderpest virus

Michael D. Baron,[1] Mildred Foster-Cuevas,[2] Jana Baron[2] and Thomas Barrett[1]

Divisions of Molecular Biology[1] and Immunology[2], Institute for Animal Health Pirbright Laboratory, Ash Road, Pirbright, Surrey GU24 0NF, UK

We have investigated the bovine immune response to heterologous proteins expressed using a recombinant rinderpest virus (RPV). A new gene unit was created in a cDNA copy of the genome of the vaccine strain of RPV, and an open reading frame inserted that encodes the polymerase ($3D^{pol}$) and parts of the capsid protein VP1 from foot-and-mouth disease virus (FMDV). Infectious recombinant RPV was rescued and shown to express the FMDV-derived protein at good levels in infected cells. The rescued virus was only slightly more attenuated in tissue culture than the original virus. Cattle infected with this recombinant generated a normal immune response to RPV, and were protected from lethal challenge by that virus. Experimental animals showed a specific delayed-type hypersensitivity response to FMDV $3D^{pol}$, similar to that seen in FMDV infection; however, no antibodies were detected recognizing either of the components of the FMDV-derived protein, nor was any proliferative response to these epitopes found in isolated peripheral blood lymphocytes from infected animals. No protection was seen against FMDV infection.

Introduction

The development of techniques for manipulating the genomes of negative-strand RNA viruses through the rescue of live virus from cDNA clones (Schnell *et al.*, 1994; Garcin *et al.*, 1995; Lawson *et al.*, 1995; Radecke *et al.*, 1995; Whelan *et al.*, 1995) has opened the way not only to investigation of the function of viral proteins and genetic elements, but also to the functional replacement of viral proteins (Mebatsion *et al.*, 1997; Schnell *et al.*, 1997; Takada *et al.*, 1997; Spielhofer *et al.*, 1998) and the expression of additional proteins by the insertion of new genes into the viral genomes (Bukreyev *et al.*, 1996; Mebatsion *et al.*, 1996; Schnell *et al.*, 1996; Hasan *et al.*, 1997; Yu *et al.*, 1997; Jin *et al.*, 1998; Moriya *et al.*, 1998; Singh & Billeter, 1999). This last type of modification has been done primarily with coding sequences for marker proteins such as chloramphenicol acetyltransferase or luciferase (Bukreyev *et al.*, 1996; Mebatsion *et al.*, 1996; Schnell *et al.*, 1996; Hasan *et al.*, 1997), but recent reports have highlighted the possibilities of using such systems for preparative biosynthesis of mammalian proteins (Yu *et al.*, 1997; Moriya *et al.*, 1998) or expressing proteins that may have effects on infected animals *in vivo* (Singh & Billeter, 1999). No limit has yet been found on the number or size of proteins that can be expressed from such recombinants; the major limiting factor is likely to be the difficulty in manipulating the full-length genome plasmids.

Rinderpest virus (RPV) is a paramyxovirus in the genus *Morbillivirus*, closely related to the causative agents of measles and canine, seal and cetacean distempers, and itself responsible for an economically highly important disease affecting cattle and wild bovids. Despite concerted eradication campaigns, the virus remains enzootic in parts of the Indian subcontinent, as well as several countries in the Near and Middle East, and East Africa, with recent outbreaks in Kenya, Tanzania, Russia and Turkey. We have developed techniques for rescuing recombinant RPV from cDNA clones (Baron & Barrett, 1997); with experimental access to the natural host of the virus, this system provides a useful tool for studying the potential for recombinant morbilliviruses to act as multivalent vaccines.

Foot-and-mouth disease (FMD) is a highly contagious disease affecting a wide range of cloven-hoofed animals, including cattle and pigs, outbreaks of which can be economically devastating. Control is achieved through inactivated virus vaccines, which provide only short-term (4–6 months) protection, and are specific for one of the seven known serotypes. In addition, these vaccines do not fully prevent the virus from establishing a persistent infection, creating carrier

Author for correspondence: Michael Baron.
Fax +44 1483 232448. e-mail michael.baron@bbsrc.ac.uk

animals even in a vaccinated herd (Salt, 1993). This contrasts with the long-lasting protection generated by a live attenuated morbillivirus vaccine such as that used for RPV. In the work presented here we have used the RBOK vaccine strain of RPV to express epitopes from structural and non-structural proteins of FMD virus (FMDV) in the hope that we would elicit a neutralizing immune response that was long lasting. The main neutralizing antibody response to FMDV is directed to the VP1 capsid protein, in particular $VP1_{141–160}$ and $VP1_{200–213}$ (Strohmaier *et al.*, 1982; Geysen *et al.*, 1984; DiMarchi *et al.*, 1986; Francis *et al.*, 1990). Peptides (DiMarchi *et al.*, 1986) and fusion proteins (Broekhuijsen *et al.*, 1986, 1987; Clarke *et al.*, 1987; Morgan & Moore, 1990) containing these regions have been shown to induce protective immune responses *in vivo*, though not consistently. Immunization with peptide FMDV15 (which contains $VP1_{200–213}$ –ProProSer– $VP1_{141–158}$ of serotype O_1Kaufbeuren), although successful in immunizing guinea pigs, only protected a minority of cattle (DiMarchi *et al.*, 1986), probably reflecting the failure of FMDV15 to generate epitopes which are presented by most bovine MHC class II haplotypes (Glass *et al.*, 1991). Addition of an appropriate source of class II epitopes to peptide FMDV15 should result in a more effective stimulation of neutralizing antibodies. Cattle infected or vaccinated with a variety of serotypes of FMDV develop $CD4^+$ T-cell responses directed to the FMDV RNA polymerase ($3D^{pol}$) (Foster-Cuevas, 1996; Collen *et al.*, 1998; Foster *et al.*, 1998) as well as serotype-cross-reactive antibodies recognizing $3D^{pol}$ (Cowan & Graves, 1966; Fernandez *et al.*, 1975; Dawe & Pinto, 1978; Polatnick & Wool, 1981; Alfonso *et al.*, 1988), indicating that this protein may contain suitable widely recognized class II epitopes. The construct we used in these studies consisted of the FMDV $3D^{pol}$ fused genetically at its carboxy terminus to peptide FMDV15.

Methods

■ **Cells and viruses.** Culture of cell lines 293, B95a and Vero, rescue of live RPV from cDNA, growth of virus stocks, determination of virus growth rate and titration of viruses was as previously described (Baron & Barrett, 1997).

■ **Molecular biology techniques and construction of plasmids.** All DNA manipulation was performed as previously described (Baron & Barrett, 1997). Plasmid pGEX-3DFMDV15 was made by amplifying the sequence of FMDV $3D^{pol}$ from plasmid pMR15 (Ryan *et al.*, 1989) using *Taq* polymerase and primers OR31 (5′ GGGGGGAATTCCGGGTTGATTGTGGAC 3′) and MF10 (5′ GGGGTGCCGGCAGGATCCACGCGGAACCAGCGCGTCACCGCACACGGC 3′), digesting with *Eco*RI and *Nae*I and ligating into similarly digested pGEX-3XUC-FMDV15 (Foster-Cuevas, 1996). The final construct was Met-[$3D^{pol}$]LeuValProArgGlySerCys[$VP1_{200–213}$]ProProSer[$VP1_{143–160}$]Cys Gly. To make pRPVins, two oligonucleotides were made, SPHINS1 (5′ TCATTATAAAAAACTTAGGATCCAAGGTCGGCGCGCCGCATG 3′) and SPHINS2 (5′ CGGCGCGCCGACCTTGGATCCTAAGTTTTTTATAATGACATG 3′). These oligonucleotides were kinased, heated to 80 °C and allowed to anneal at a concentration of 9·4 μM. Approximately 23 fmol of the dsDNA fragment was ligated into *Sph*I-digested, alkaline phosphatase-treated pMDBN2F (Baron & Barrett, 1997). Clones that had acquired the *Asc*I site were sequenced. From one clone that had the correct sequence, and the insert in the correct orientation, the *Cla*I–*Sun*I fragment, containing all of the N, P, M genes and most of the F gene, was removed and ligated into the *Sun*I–*Cla*I fragment of pMDBRPV (Baron & Barrett, 1997), containing the rest of the genome.

To make pRPV-FMD, the 3DFMDV15 region of pGEX3DFMDV15 was amplified using *Pfu* polymerase (Stratagene) essentially according to the manufacturer's instructions. The primers used were 5′ CCCATGGCGCGCCATGGGGTTGATTGTGGACA 3′ (FMD3DF) and 5′ CCCTAGGCGCGCCTCTATTATCCGCATGGTA 3′ (FMD3DR). The amplified product was digested with *Asc*I, purified in a low melting point agarose gel, and ligated into *BssH*II-cut, alkaline phosphatase-treated pRPVins. One correct clone was selected and, after sequencing the insert, used for virus rescue.

■ **Antibodies, immunoprecipitation and immunofluorescence.** Rabbit antisera recognizing the RPV P protein (Baron & Barrett, 1997) were raised against bacterially expressed glutathione *S*-transferase (GST) fusion proteins. The rabbit anti-RPV N antiserum was raised against a mixture of synthetic peptides representing RPV-$N_{402–424}$, RPV-$N_{425–451}$, RPV-$N_{452–476}$ and RPV-$N_{477–501}$. Mouse monoclonal antibody recognizing the RPV M protein was prepared by standard techniques from mice immunized with partially purified RPV (M. Fleming & T. Barrett, unpublished). Rabbit anti-FMDV $3D^{pol}$ was the kind gift of T. Collen and A. Corteyn of this institute. Mouse monoclonal antibody B2 recognizing the FMDV15 peptide was the generous gift of E. Brocchi, Inst. Zooprofilatico Sperimentale Lombardia Emilia, I-25124 Brescia, Italy. Mouse monoclonal antibody 2-1 recognizing the RPV P protein was the kind gift of M. Sugiyama, Dept. of Veterinary Public Health, Faculty of Agriculture, Gifu University, Gifu 501-11, Japan.

For immunoprecipitation studies, cells in six-well plates were infected with RPV for 2 days, incubated for 1 h in Eagle's medium without methionine or cysteine, then for 2 h in 0·5 ml of the same medium containing 5–10 μCi Pro-mix (Amersham Pharmacia), a mixture of amino acids containing [^{35}S]methionine and [^{35}S]cysteine. The medium was removed and the cells lysed in 0·5 ml lysis buffer (1%, v/v, Nonidet-P40, 50 mM Tris–HCl, pH 7·5, 0·5 M NaCl, 5 mM EDTA, 0·5 mM PMSF, 2·5 mM iodoacetamide, and 100 ng/ml each of leupeptin, pepstatin, antipain and chymostatin). The lysate was incubated first with non-immune rabbit serum and protein A–Sepharose (Amersham Pharmacia) for 1–2 h at 4 °C, centrifuged to remove the protein A–Sepharose, and then incubated with the specific antiserum and protein A–Sepharose overnight at 4 °C. Where the primary antibody was a mouse monoclonal, 0·5 μl of rabbit anti-mouse IgG (Dakopatts) was added in addition. Sepharose pellets were washed and samples prepared for SDS–PAGE as described (Baron & Garoff, 1990).

SDS–PAGE was performed using the buffer system of Laemmli (1970); gels were fluorographed using sodium salicylate (Chamberlain, 1979).

For immunofluorescence studies, infected cells growing on glass coverslips were fixed 2 days post-infection and stained essentially as described (Timm *et al.*, 1983); secondary antibodies (FITC and Texas Red conjugates) were obtained from Molecular Probes, Inc.

Fluorographs of gels and photographs of immunofluorescently stained cells were scanned using a Linotype Hell Saphir or a Kodak DCS200 digital camera and imported into Adobe Photoshop 4.01. Images were laid out and text added using Quark Express 3.32 and printed on a Kodak XLS8600 PS dye-sublimation printer.

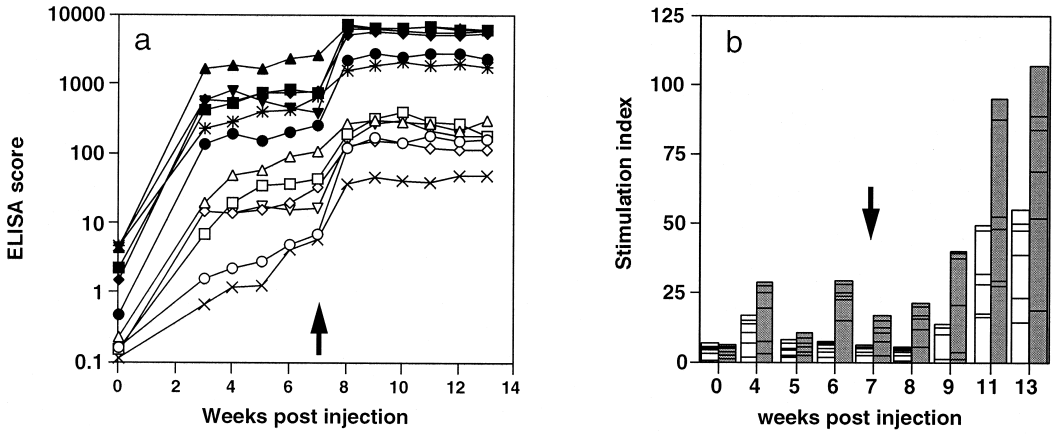

Fig. 1. Immune responses in cattle to bacterially synthesized GST–3D15 fusion protein. Six cattle were injected with fusion protein as described in Methods, with a second injection given at 7 weeks [arrow in (*a*) and (*b*)]. At the times shown, serum and PBLs were prepared. (*a*) Direct binding ELISA was used to measure the relative amount of anti-GST (open symbols) and anti-GST–3D15 (closed symbols) present in the serum. (*b*) PBL proliferation induced by bacterially expressed fusion proteins. Cells from the individual animals were exposed to GST (open bars) or GST–3D15 (filled bars) and the separately determined stimulation indexes plotted stacked together.

3341 ACATAGCTGATCTCCTCGCTTCCCCTCAGCTTAGCTACAGCTTGCATGTC

3391 ATTATAAAAAACTTAGGATCCAAGGTC*GGCGCGCCGC*ATGCTCATTATAA
End of P gene — Start of new gene End of new

3441 AAAACTTAGGAGCAAAGCATAACGCCTCGCACTCCCAACATGGCAGAAAT
gene — Start of M gene

Fig. 2. Sequence of the modified region of the RPV genome in plasmid pRPVins. The new sequence inserted into the genome is highlighted (boxed). The gene start and end sequences of the final construct are marked, as are the original and new intergenic trinucleotides (underscored) and the unique *Asc*I site (italics). The sequence is shown in antigenome sense, with numbering from the start of the antigenome.

■ **Preparation of purified fusion proteins and viral antigen.** Bacterially expressed proteins for immunogenicity studies (GST, GST–3DFMDV15) were expressed in *E. coli* JM109 and batch purified on glutathione–agarose (Amersham Pharmacia) according to the manufacturer's instructions. For delayed type hypersensitivity (DTH) assays, the antigens were purified by preparative SDS–PAGE. Partially purified RPV proteins were prepared from RPV-infected Vero cells as described (Barrett *et al.*, 1989); uninfected Vero cells were processed in parallel to provide a suitable negative control. Purified FMDV O_1 total antigen, lot 6.0.142.C, was obtained from Rhône-Merieux (now Merial).

■ **Animal studies.** To study the immunogenicity of the 3DFMDV15 fusion protein in cattle, six animals were injected subcutaneously with 3 mg each of purified fusion protein in Freund's complete adjuvant, and boosted at 7 weeks with a further 2 mg of protein in Freund's incomplete adjuvant. Blood was taken at weeks 0, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 13 for determination of serum antibodies and isolation of peripheral blood lymphocytes (PBLs).

For studies with recombinant RPV, animals were infected with approximately 10^4 $TCID_{50}$ units of virus in the right shoulder. Blood was taken at 3 to 4 day intervals for serology and isolation of PBLs. At 3 to 4 weeks post-infection, DTH tests were made (as described in Foster *et al.*, 1998), after which the appropriate challenge was performed with 10^4 $TCID_{50}$ units of the virulent Saudi/81 strain of RPV or with 10^5 $TCID_{50}$ units of FMDV strain O_1 Lausanne. For the DTH tests, 20 μg of bacterially expressed GST or GST–3D, or 10 μg of RPV-infected/uninfected Vero cell protein, or 7·5 μg of FMDV total antigen was used in 100 μl PBS.

■ ***In vitro* immunological assays.** PBLs were isolated from bovine sera as described in García-Valcarel *et al.* (1996). For proliferation studies, 10^5 cells were incubated with antigen (0·125 μg) in triplicate for 5 days. They were then pulsed with [*methyl*-^{3}H]thymidine (NEN) for 6 h before harvesting onto glass-fibre filter mats and counting. The results are expressed as the stimulation index, the ratio of the ^{3}H incorporated into cells incubated with antigen to that incorporated into cells incubated with medium alone.

Indirect ELISAs for measuring antibodies recognizing GST fusion proteins were performed as follows. Purified GST or fusion protein (250 ng per well) was used to coat 96-well microtitre plates (Nunc). After blocking in PBS–0·05% (v/v) Tween 20–3% (w/v) BSA, plates were incubated with duplicate threefold serial dilutions of test sera, starting from 1/100, for 1 h at 37 °C. The plates were further incubated with horseradish peroxidase-conjugated rabbit anti-bovine IgG (1/1000, Dakopatts), then developed with *o*-phenylenediamine dihydrochloride (OPD) solution (2·5 mg/ml OPD in 0·15 M citrate–phosphate buffer, pH 5 containing 1/1000 dilution of 20 vol. H_2O_2). The absorbance was measured at 492 nm. To calculate the ELISA score, readings from the linear range of dilutions were extrapolated to a notional absorbance at 1/100 dilution for an unsaturable assay.

Results

In preliminary studies, the $3D^{pol}$–FMDV15 (3D15) fusion protein was expressed in *E. coli* as a carboxy-terminal fusion with GST and then injected into cattle. Clear immune responses

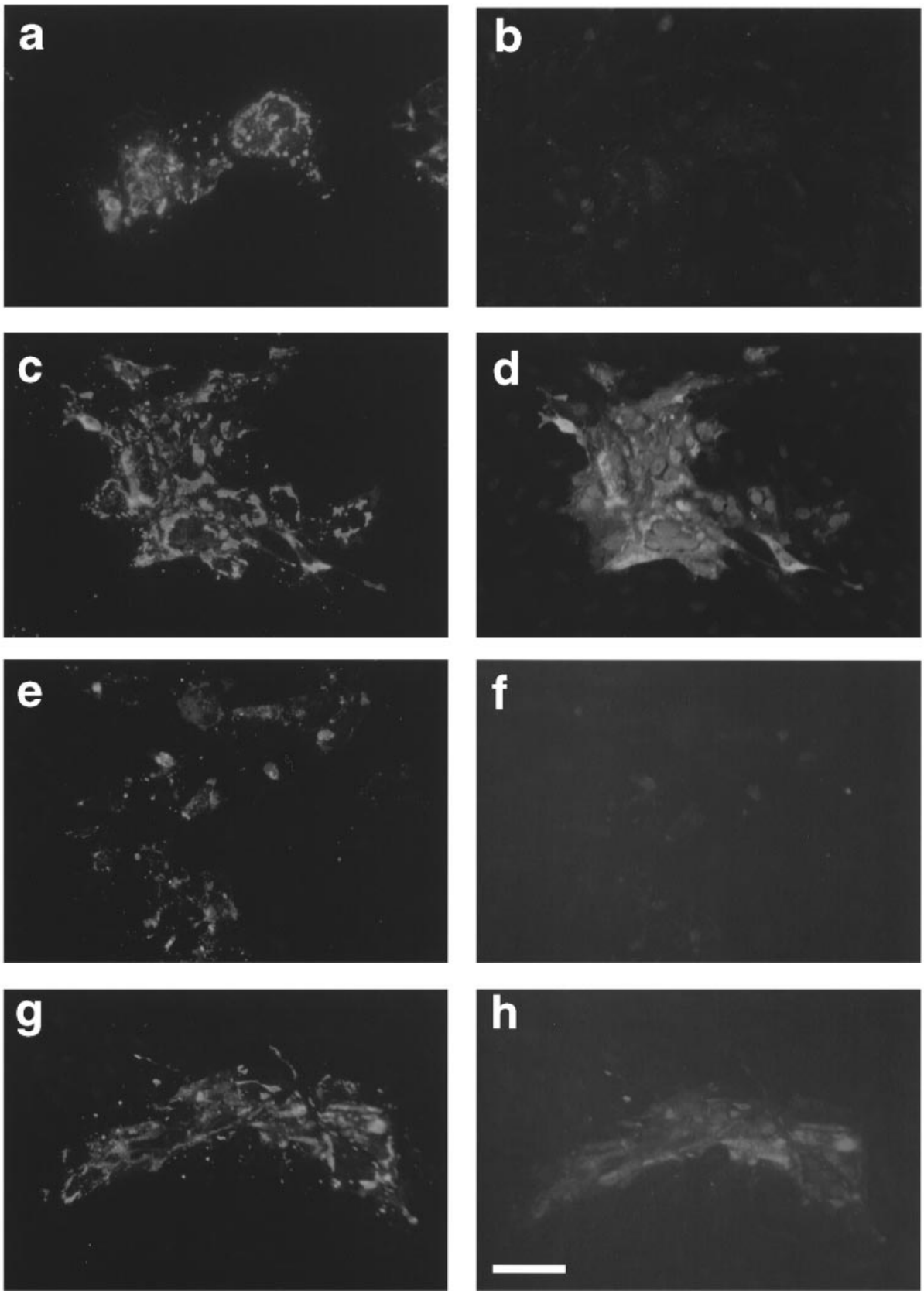

Fig. 3. For legend see facing page.

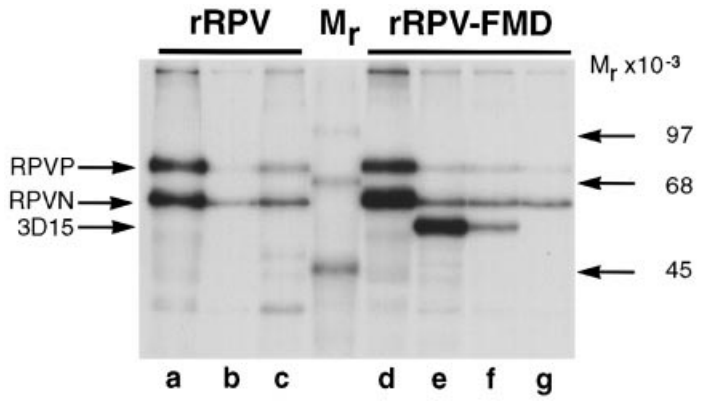

Fig. 4

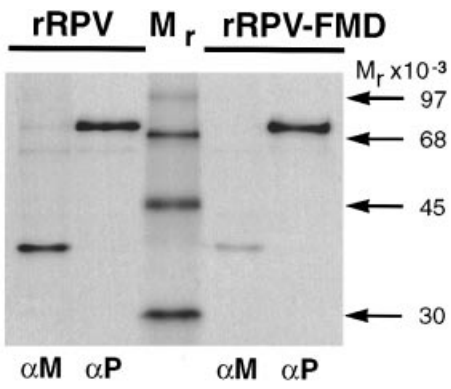

Fig. 5

Fig. 4. Expression of 3D15 fusion protein in rRPV-FMD-infected cells. B95a cells were infected with rRPV (*a–c*) or rRPV-FMD (*d–g*) and labelled with ^{35}S Pro-mix, lysed and aliquots of lysate extracted with various antibodies as described in Methods: (*a*), (*d*) rabbit anti-RPV-N plus rabbit anti-RPV-P; (*b*), (*e*) rabbit anti-3D^{pol}; (*c*), (*f*) mouse anti-FMDV15; (*g*) lysate from (*e*) re-extracted with anti-FMDV15. Immunoprecipitated proteins were analysed on a 10% SDS–PAGE gel and the gel fluorographed.

Fig. 5. Effect of gene insertion of expression of downstream genes. B95a cells were infected, labelled and lysed as in Fig. 4. Aliquots of lysate were extracted with the indicated antibodies (αM, mouse anti-RPV M monoclonal; αP, mouse anti-RPV P monoclonal) and the immunoprecipitated proteins analysed on a 12% SDS–PAGE gel, and the gel fluorographed.

(Fig. 1) to the FMDV-derived elements of the fusion protein were found, although the boost injection given at week 7 was required for all animals to show anti-3D15 antibodies and proliferative responses. GST–3D gave a response intermediate between GST and GST–3D15, showing that both the 3D^{pol} and FMDV15 portions of the fusion protein were immunostimulatory (not shown).

In order to express the fusion protein from RPV, a new gene had to be created in the viral genome. Transcription of mRNAs from the genomes of nonsegmented negative-strand RNA viruses appears to be driven from a single promoter at the 3′ end of the genome, and levels of transcription are dependent on the position of the gene along the genome (Pringle & Easton, 1997); those genes nearest the promoter are expressed at higher levels (Wertz *et al*., 1998). As a compromise between our desire for strong expression of the fusion protein and the need not to suppress expression of normal viral proteins to the point where an already attenuated virus became non-viable, we chose to introduce the new gene immediately downstream of the P gene. This should preserve normal levels of the nucleocapsid (N) and phospho (P) proteins, which are required in large amounts for genome replication.

The new 'gene' consisted of new gene stop signal, intergenic trinucleotide and a minimal gene start signal, followed by a unique restriction site (*Asc*I), all inserted in the 3′ untranslated region (UTR) of the P gene. The gene start and end sequences of morbilliviruses are not fully conserved (Baron & Barrett, 1995), and the exact roles played by individual bases have not been defined. We therefore duplicated existing sequences from the RPV genome for the new 'gene', the gene stop/polyadenylation signal from the P gene and the gene start from the H gene. The sequence of the modified region of the genome (in mRNA sense) is shown in Fig. 2. The total length of the inserted nucleotides was 42, keeping the genome length a multiple of six, as appears to be necessary for replication of some paramyxoviruses, including Sendai virus (Calain & Roux, 1993) and morbilliviruses (Radecke *et al*., 1995; Baron & Barrett, 1997). An open reading frame cloned into the *Asc*I site should be transcribed and polyadenylated and, if it has a suitable translation initiation site (Kozak, 1984), will be translated into the desired protein.

The 3D15 construct was amplified by *Pfu* polymerase in a PCR, introducing the translation initiation signal and appropriate restriction sites in the primers. This DNA was then cloned into the modified genome plasmid and the sequence checked. Again, the total length of the inserted nucleotides was a multiple of six. Virus rescue was performed as previously described (Baron & Barrett, 1997). The virus containing only the extra gene start and stop signals was never rescued, despite many transfections; the same genome plasmid, containing the full 3D15 insertion, was rescued as virus at the first attempt.

Cells infected with the rescued virus (rRPV-FMD) showed clear staining by antibodies to RPV proteins, to 3D^{pol} and to FMDV15 (Fig. 3). Immunoprecipitation of radiolabelled

Fig. 3. Expression of 3D15 in rRPV-FMD-infected cells. Vero cells were infected with rRPV (*a*, *b*, *e*, *f*) or rRPV-FMD (*c*, *d*, *g*, *h*), fixed at 2 days post-infection and labelled with mouse anti-RPV P protein (*a*, *c*) and rabbit anti-3D (*b*, *d*) or rabbit anti-RPV N protein (*e*, *g*) and mouse anti-FMDV15 (*f*, *h*). Bound antibody was visualized with FITC-labelled goat anti-rabbit IgG and Texas Red-labelled goat anti-mouse IgG, as described in Methods. Scale bar, 50 μm.

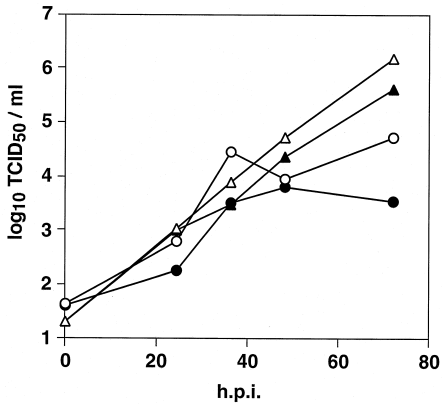

Fig. 6. Growth of recombinant RPV in tissue culture. The total (cell associated and medium) virus present in cultures infected with rRPV (open symbols) or rRPV-FMD (closed symbols), in either B95a cells (triangles) or Vero cells (circles) was measured as described in Methods. Each line is the mean of two separate experiments.

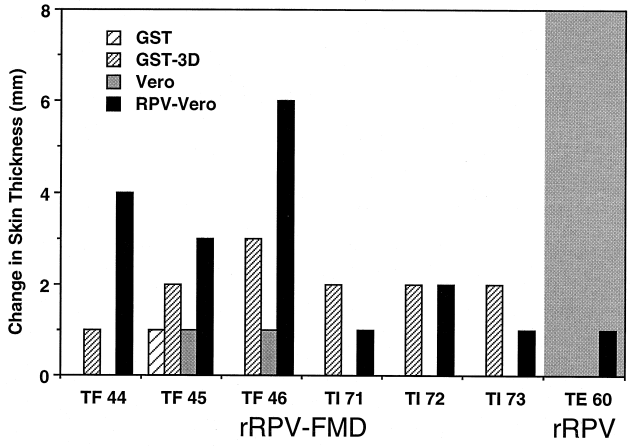

Fig. 7. Delayed-type hypersensitivity responses in cattle infected with rRPV (TE60) or rRPV-FMD (all other animals) 3 weeks prior to test. Antigen suspended in 100 μl PBS was injected intradermally as described in Methods, and the skin thickness measured 2 days later. The response is expressed as the increase in skin thickness over a control region of skin injected only with PBS.

proteins synthesized in infected cells showed that the fusion protein was synthesized at a level similar to that of the viral P protein and could be precipitated by anti-3D^{pol} or anti-FMDV15 (Fig. 4), though somewhat poorly by the latter antibody. We also found that, as expected, the insertion of the extra gene had altered the relative levels of P and M made in infected cells (Fig. 5), and this alteration may be the cause of the slightly decreased growth rate of the virus in culture (Fig. 6).

Initially, six cattle were infected with rRPV-FMD, and one with unmodified rRPV. Three animals given rRPV-FMD and the rRPV control were challenged with virulent RPV 3 weeks post-infection and all were completely protected. The three remaining rRPV-FMD-infected animals were challenged with FMDV (strain O_1), but no protection was found. Prior to challenge with either RPV or FMDV, all six experimental animals showed clear DTH responses to 3D^{pol} (Fig. 7), though not to FMDV (not shown), suggesting there was no recognition of the FMDV15 peptide. No proliferative response in PBLs to 3D^{pol}, FMDV15 or FMDV was detectable, nor were any antibodies recognizing 3D^{pol} or FMDV detected, either by ELISA or Western blot, although serum anti-RPV antibodies were detected as normal (not shown).

Discussion

We have shown that recombinant RPV can be constructed that produces large quantities of expressed heterologous protein in tissue culture. The virus was only slightly impaired in growth, and functioned normally as a vaccine against RPV. An interesting observation was that the genome with a whole new gene, including open reading frame, (pRPV-FMD) could be rescued without problems, whilst we have continually failed to rescue the genome with the much smaller inserted sequence containing only the gene start and end sequences (pRPVins). This cannot be due to an extra, deleterious, mutation in pRPVins, since the same plasmid stock was used to construct pRPV-FMD. We have since shown that the addition of an extra 66 bases at the *Asc*I site of pRPVins renders it fully rescuable (M. D. Baron, unpublished), and this may have implications for the minimum length of a gene in RPV. If the new gene stop in RPVins was too close to the preceding gene start, and was not recognized by the viral polymerase, the resultant bicistronic mRNA would be the only transcript of the viral M gene. It would contain an AUG codon upstream of, and out of frame with, the normal M protein start codon, albeit in a poor context (CGC**ATG**C), and this might have very serious consequences for virus viability. A form of measles virus (MV) has been rescued with no M gene (Cathomen *et al.*, 1998), and this virus is severely attenuated, and our own observations have suggested that the more attenuated the virus, the lower the frequency of rescue events in transfected cells. If the hypothetical virus RPVins did not express M protein, and was equally attenuated and hard to rescue, it is probable that we would have had to transfect 10 or 100 times as many cells in order to isolate the virus.

Although the heterologous protein expressed from the recombinant RPV-FMD was composed of regions of FMDV proteins known to be immunogenic in FMDV infection, and the fusion protein was itself shown to be immunogenic when injected directly into animals, only a minimal immune response was found *in vivo* when this protein was expressed using the recombinant RPV vaccine. There are several possible reasons why the observed response was so poor.

One explanation could be that RPV, like MV, is a lymphotropic virus. MV infection is immune suppressive, and we have observed a similar suppressive effect of RPV on lymphoid cell replication in culture (J. Heaney, T. Barrett &

S. L. Cosby, unpublished). However, the suppression *in vivo* brought about by MV is characterized by a decrease in mitogen sensitivity and DTH responses, and we found normal responses to concanavalin A in PBLs from RPV-vaccinated animals (data not shown), and good DTH responses to both RPV and the FMDV $3D^{pol}$. It is unlikely, therefore, that the animals were immune suppressed.

Alternatively, the expressed protein was not being correctly presented to elicit a humoral response. Cytoplasmically expressed proteins would be expected to generate peptides that would primarily enter the MHC class I presentation pathway after degradation by the proteasome (Driscoll *et al.*, 1993). However, there is clear evidence that peptides from cytoplasmically synthesized proteins can cross over into the MHC class II pathway (Brooks & McCluskey, 1993; Aichinger & Lechler, 1995). In addition, proteins released from dead cells can be taken up and degraded by specialist antigen-presenting cells, thereby entering the normal exogenous route to class II presentation. A polyepitope fusion protein expressed from vaccinia virus generated both cytotoxic T-cell and antibody responses to various components (An & Whitton, 1997), showing that peptides from a single protein can enter both pathways. In our case, the generation of the Th1-dependent DTH reaction showed that peptides from $3D^{pol}$ were entering the class II pathway. In fact, the anti-$3D^{pol}$ DTH response observed after infection with rRPV-FMD (6/6) was at least as good as that observed for single dose infection of cattle with FMDV itself [9/12 (Foster *et al.*, 1998)].

On the other hand, FMDV infection invariably gives rise to detectable anti-$3D^{pol}$ antibodies (Berger *et al.*, 1990), which were not found in the rRPV-FMD-infected animals. This difference may reflect the different cytopathologies of the (attenuated) RPV and normal FMDV: whilst the latter is highly lytic, at least in cultured cells, the former is not. Coupled with the low levels of replication achievable by a vaccine strain, it is possible that only a very small amount of intact 3D15 protein is being released in rRPV-FMD-infected animals to act as a target for the B cell repertoire. A recombinant virulent strain would doubtless express much higher levels of heterologous protein, but this would rather defeat the object of the exercise.

If the level of expression and release from the cell of the expressed non-RPV protein are the limiting factors, it will be necessary to improve the efficiency of delivery to the MHC class II pathway in order to maximize the response and improve the efficacy of this system as a vaccine delivery tool. Increased release of intact protein is also likely to be helpful. We have already found that adding a signal peptide to a protein, causing it to be translocated into the endoplasmic reticulum and secreted from infected cells, increases the serum antibody response, though not to 100% (E. P. Walsh, M. D. Baron & T. Barrett, unpublished). Further increases in immune stimulation may be achieved by anchoring the expressed protein in the cell membrane, targeting it either to the surface of the cell or to the endosomal/lysosomal compartment in which MHC class II molecules are loaded (Peters *et al.*, 1991; Marks *et al.*, 1995), and we are investigating these possibilities in our current research.

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