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Development of a genetically marked recombinant rinderpest vaccine expressing green fluorescent protein

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In order to effectively control and eliminate rinderpest, a method is required to allow serological differentiation between animals that have been vaccinated and those which have recovered from natural infection. One way of doing this would be to engineer the normal vaccine to produce a genetically marked rinderpest virus (RPV) vaccine. We constructed two modified cDNA clones of the RPV RBOK vaccine strain with the coding sequence of the green fluorescent protein (GFP) gene inserted as a potential genetic marker. RPVINS-GFP virus was designed to produce independent and high level expression of GFP inside infected cells, whilst the GFP expressed by RPVSIG-GFP virus was designed to be efficiently secreted. Infectious recombinant virus was rescued in cell culture from both constructs. The effectiveness of these viruses in stimulating protective immunity and antibody responses to the marker protein was tested by vaccination of cattle and goats. All of the vaccinated animals were completely protected when challenged with virulent virus: RPV in cattle or peste-des-petits ruminants virus in the goats. ELISA showed that all of the animals produced good levels of anti-RPV antibodies. Three of the four cattle and the two goats vaccinated with RPVSIG-GFP produced detectable levels of anti-GFP antibodies. In contrast, no anti-GFP antibodies were produced in the four cattle and two goats vaccinated with RPVINS-GFP. Therefore, secretion of the GFP marker protein was absolutely required to elicit an effective humoral antibody response to the marker protein.

Introduction

Rinderpest is a severe disease of wild and domestic ruminants, characterized by high morbidity and mortality rates, which is of great economic importance in affected African and Asian countries. Rinderpest virus (RPV) is a nonsegmented negative-strand RNA virus which is classified in the genus *Morbillivirus* of the family *Paramyxoviridae*. There is great variation in the virulence and pathogenicity of different field isolates of RPV although they are all of a single serotype (Taylor, 1986). RPV is genetically and antigenically very closely related to other viruses in the genus *Morbillivirus*, which include measles virus and peste-des-petits ruminants virus (PPRV).

An international campaign is currently underway with the aim of global eradication of rinderpest by the year 2010 (Rweyemamu & Cheneau, 1995; Taylor *et al.*, 1995; Anon., 1998). In the final stages of this campaign countries will have to stop vaccination and show that in the absence of herd immunity there is no hidden disease resulting from circulating mild strains of the virus. However, a rinderpest vaccine will still be required in the transition phase of local eradication campaigns and for emergency vaccination during isolated outbreaks of the disease. The most commonly used RPV vaccine is the Plowright attenuated RBOK strain, which was derived by multiple passage in cell culture of the virulent Kabete 'O' strain (Plowright & Ferris, 1962). The RBOK vaccine is safe and effective, providing complete and lifelong protection from rinderpest with a single inoculation (Plowright & Ferris, 1962; Plowright, 1984). Because all strains of the virus, including the vaccine, are of one serotype, it is not possible to distinguish serologically between cattle which have recovered from a natural infection and those which have been vaccinated. An RPV vaccine which could be serologically distinguished from natural virus infection would therefore be of great value during the transition phase of the eradication campaign.

Since it is possible to rescue recombinant RBOK virus from a cDNA clone (Baron & Barrett, 1997), and as the RBOK

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vaccine has been proven to be safe and effective, it is logical to use this strain for the development of a genetically marked rinderpest vaccine. Many full-length negative-strand RNA viruses have been produced which stably express foreign genes, such as simian virus 5 expressing green fluorescent protein (He *et al.*, 1997), and measles virus expressing hepatitis B virus antigens (Singh *et al.*, 1999). Therefore, it should be possible to produce a recombinant RBOK vaccine carrying a foreign gene as a genetic marker. It should then be possible to distinguish serologically between vaccinated and naturally infected animals. We have created a gene cassette between the P and M genes of RPV RBOK, and shown that non-RPV proteins can be expressed at high levels from this position (Baron *et al.*, 1999). We introduced the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* (reviewed in Tsien, 1998), into full-length cDNA clones of the RBOK vaccine strain to produce genetically marked vaccines which were tested in animal vaccination trials.

Methods

■ **Cell culture and viruses.** Culture of 293, B95a and Vero cell lines, virus growth, determination of virus growth rate and titrations were carried out as described previously (Baron *et al.*, 1999).

■ **Construction of pRPVINS-GFP and pRPVSIG-GFP.** The construction of pRPVINS, in which new gene start and stop signals, bracketing a unique *Asc*I restriction enzyme cleavage site, have been inserted into the RPV cDNA clone, has been described previously (Baron *et al.*, 1999). To make pRPVSIG, two oligonucleotides were prepared, which when annealed made a 66 bp fragment (5′ CGCGCTACCATGGGTCGTAAACTGCTGCTGCTGCTGCTGCTGCTGCTGCCAACCGCACGTGGG 3′) with *Asc*I compatible ends (underlined). This construct was ligated into *Asc*I-cut pRPVINS, and clones containing a single copy, inserted in the correct orientation, were identified by restriction enzyme mapping and sequencing. The GFP sequence was inserted into these modified full-length cDNA clones of RPV. The GFP open reading frame (ORF) was obtained from pEGFP-C1 (Clontech) by PCR with Pfu polymerase (Stratagene) using the primers 5′ CGTAGCGCGCAATGGTGAGCAAGGGCGAGGAGCTGT 3′ (*Bss*HII site underlined) and 5′ ATTAGGCGCGCCATCATCACTTGTACAGCTCGTCCATGCCGA 3′ (*Asc*I site underlined). The PCR product was digested with *Bss*HII, and as *Bss*HII and *Asc*I restriction sites have compatible ends, the 732 bp fragment was ligated into the *Asc*I site in both pRPVINS and pRPVSIG to produce the pRPVINS-GFP and pRPVSIG-GFP plasmids, respectively. In the latter clone, the GFP ORF is in-frame with the signal sequence (Von Heijne, 1983; Baron *et al.*, 1992), and cleavage by the endoplasmic reticulum signalase will release the full GFP. Both recombinant RPV viruses were designed to obey the rule of six for genome length (Calain & Roux, 1993). The identity of the GFP ORF inserted into the pRPVINS and pRPVSIG *Asc*I restriction site was confirmed by sequencing.

■ **Transfection and recovery of recombinant viruses.** 293 cells were infected with vaccinia virus MVA-T7at an m.o.i. of 2. Transfections were performed using Fugene 6 (Roche) according to the manufacturer's instructions. For each well in a six-well 35 mm plate, 20 µl of Fugene 6 was added to 180 µl Opti-MEM I (Gibco) and incubated for 5 min. The RPV N, P, L plasmids and genome plasmid mix was prepared by combining 1 µg pKS-N1, 1 µg pKS-P, 0·05 µg pGEM-L, and 2 µg of either pRPVINS-GFP, pRPVSIG-GFP or pRPVII (positive control) plasmids. Diluted Fugene 6 was added to the plasmids and the Fugene 6/plasmid mix was incubated at room temperature for 15 min. The MVA-T7/Opti-MEM I was removed from the cells and replaced with 2 ml DMEM medium, and then approximately 200 µl of Fugene 6/plasmid mix was added to the cells in each well. Cells were incubated at 37 °C for 3 days, and then virus was extracted as previously described. Virus supernatant (500 µl) was used to infect B95a cells. The cultures were incubated until RPV cytopathic effect (CPE) was visible, and then extracted virus was used to infect Vero cells.

■ **RT–PCR of viruses.** B95a cells were infected with virus at an m.o.i. of 0·01. Cultures were grown at 37 °C for 4 days until CPE was visible. RNA was recovered from these cells using Trizol reagent (Gibco) according to the manufacturer's instructions. RT–PCR was carried out as described previously (Forsyth & Barrett, 1995). RPV-specific primers bracketing the *Asc*I insertion site for the GFP ORF were used in the PCR reactions. Primer 5′ CGCAGTGTGATCCGTTC 3′ (RPV-P6) primes in the P gene and anneals to nucleotides 3297–3313 of the antigenome in the recombinant RPVII virus (GenBank accession no. Z30697). Primer 5′ TCTTGCATCTGGATCTC 3′ (RPV-M2) primes in the M gene and anneals to nucleotides 4437–4451 of the antigenome in the recombinant RPVII virus.

■ **Radioimmunoprecipitations.** Radioimmunoprecipitations were carried out using virus-infected B95a cells as previously described (Baron *et al.*, 1999) with the following modifications: the antibodies used were 0·5 µl rabbit anti-GFP polyclonal antibody (Clontech) or 0·2 µl mouse anti-RPV-P 2-1 monoclonal antibody (Baron *et al.*, 1999) together with 0·5 µl rabbit anti-mouse antibody (Dakopatts).

■ **Immunofluorescence microscopy.** This was done as described previously (Baron *et al.*, 1999). GFP was detected both by GFP fluorescence and with anti-GFP antibody at a concentration of 1:500. Anti-RPV-P antibody was used at a concentration of 1:1000. Primary antibodies were detected with Texas red-conjugated goat anti-rabbit IgG and Marina Blue-conjugated goat anti-mouse IgG (Molecular Probes), respectively.

■ **Animal vaccination.** RPVINS-GFP and RPVSIG-GFP viruses were each used to vaccinate four 1-year-old Friesian cattle and two 1-year-old goats, and four cattle were vaccinated with the RPVII virus as controls. The cattle were challenged with the RPV-Saudi 1/81 strain at 4 weeks post-vaccination. The goats were challenged with the PPRV-Ivory coast/89 strain (gift of A. Diallo, CIRAD/EMVT, France) at 4 weeks post-vaccination for RPVINS-GFP or 3 weeks post-vaccination for RPVSIG-GFP. Vaccines and challenge viruses were administered diluted in PBS by subcutaneous injection using a dose of 10^4 $TCID_{50}$ per animal.

Blood and eye swabs were collected on specific days. Eye swabs were stored at −20 °C for RNA extraction. Peripheral blood leukocyte (PBL) counts were made with a haemocytometer. PBLs were isolated from blood for virus isolation and virus detection by RT–PCR as previously described (Baron *et al.*, 1999). The presence of virus in leukocytes and lachrymal secretions was determined by culture with B95a cells or by RT–PCR. PBLs (from a 10 ml blood sample) or a swab were added to 1 ml PBS and freeze–thawed at −70 °C. The sample was clarified by centrifugation and the supernatant added to 10^6 B95a cells in a flask. For RT–PCR, RNA was extracted directly from swabs or from pelleted PBLs isolated from a 10 ml blood sample using Trizol. RT–PCR of these samples was carried out as described previously (Forsyth & Barrett, 1995) using morbillivirus universal primers (N1 and N2), RPV-specific primers (RPV-F3B and RPV-F4D), and bovine actin-specific primers (BA1 and BA2) as an RNA-positive control.

■ **ELISA tests.** Animal serum samples were prepared from coagulated blood. The anti-RPV-H antibody response was detected using the rinderpest competitive ELISA (Anderson & McKay, 1994). The anti-GFP antibody response was detected using an indirect ELISA test carried out as follows: ELISA plates were coated with 50 µl of recombinant GFP (Clontech) at a concentration of 0·5 µg/ml in PBS. After incubation at 37 °C for 1 h, the plates were washed three times in PBS and the test sera were added at a dilution of 1:5 in blocking buffer (5%, w/v, Marvel milk powder; 0·1%, v/v, Tween 20 in PBS). Known positive and negative sera were included as controls. Plates were incubated for 1 h, washed and then anti-species (anti-bovine or anti-sheep) horseradish peroxidase-labelled enzyme conjugates (ICN) were added at a dilution of 1:3000 in blocking buffer. After final incubation and washing, substrate/chromogen (hydrogen peroxide/*o*-phenylenediamine) was added to all wells. The colour was allowed to develop for 10 min and the reaction stopped by addition of 1 M H_2SO_4. Plates were read spectroscopically at a wavelength of 492 nm using a Titertek Multiskan Plus ELISA reader. The results were expressed as OD_{492} units and values three times greater than the prevaccination values were considered positive.

Results

Rescue of recombinant viruses from cloned DNA

We have previously described the introduction of a new gene into RPV RBOK (Baron *et al*., 1999). Since the construct was designed to allow the insertion of any ORF at the unique *Asc*I site, it was straightforward to amplify the GFP ORF, introducing *Bss*HII sites (which have *Asc*I compatible ends) at each end, and insert this into pRPVINS to make pRPVINS-GFP. After constructing pRPVSIG, the GFP ORF was inserted into the *Asc*I site as for pRPVINS-GFP to give pRPVSIG-GFP. Schematic representations of the cDNA constructs which encode the RPVINS-GFP and RPVSIG-GFP virus genomes are shown in Fig. 1. RPVINS-GFP was designed to achieve independent and high level intracellular expression of GFP in infected cells, and RPVSIG-GFP was designed to efficiently secrete GFP.

The RPVINS-GFP and RPVSIG-GFP recombinant viruses were rescued in cell culture from the pRPVINS-GFP and pRPVSIG-GFP cDNA clones, respectively, using the previously established technique (Baron & Barrett, 1997). When rescued, both viruses produced syncytia, the characteristic CPE for RPV in cell culture. The viruses were grown in Vero cells to produce vaccine stocks. The CPE observed in infected B95a and Vero cell cultures was identical to that of the standard RBOK vaccine. To confirm that the rescued viruses were the correct recombinants, RT–PCR of viral RNA was carried out using the RPV-P6 and RPV-M2 primers, which bracket the *Asc*I insertion site for GFP (Fig. 2). The PCR products detected corresponded to the expected size of 1155 bp for RPVII, 1929 bp for RPVINS-GFP and 1995 bp for RPVSIG-GFP. RT–PCR of the same viral samples without using reverse transcriptase failed to produce any PCR product (data not shown), indicating that the PCR products originated from viral RNA and not from the transfectant plasmids. The PCR products from the RPV-P6 and RPV-M2 primer reactions were digested with *Asc*I to confirm the presence of the restriction site and to distinguish between the two viruses. As expected, the RPVII PCR product was not cleaved when digested with *Asc*I due to the absence of the restriction site in the virus genome. Digestion with *Asc*I of the PCR products for the other two viruses yielded the correct sized bands corresponding to the expected sizes of 965 bp and 964 bp (appear as a single band) for RPVINS-GFP, and 1031 bp and 964 bp for RPVSIG-GFP.

Comparison of vaccine growth kinetics

The growth characteristics of the RPVINS-GFP and RPVSIG-GFP viruses were compared to the standard RBOK vaccine virus. Growth curves for each of these viruses grown in Vero cells are shown for comparison in Fig. 3. The initial growth rates of the RPVINS-GFP and RPVSIG-GFP viruses were slightly slower than that of RBOK, although all three viruses produced similar titres by 48 h. The slightly reduced growth rates of RPVINS-GFP and RPVSIG-GFP were expected because they have larger genomes than the RBOK virus, and since the insertion of another gene into the genome between the P and M genes results in reduced levels of expression of the downstream M, F, H and L genes (Wertz *et al*., 1998; Baron *et al*., 1999). However, we have found that for vaccine production in Vero cell cultures, both RPVINS-GFP and RPVSIG-GFP viruses can be grown to titres (10^5–10^6 $TCID_{50}$/ml) similar to that of the RBOK vaccine (data not shown).

Analysis of GFP expression and secretion

The RBOK vaccine is primarily lymphotropic *in vivo*, infecting and replicating in leukocytes, although other tissues are infected (Taylor & Plowright, 1965; Wohlstein *et al*., 1993). Therefore, the lymphoblastoid B95a cell line (Kobune *et al*., 1990, 1991) provided an appropriate *in vitro* model for virus gene expression and secretion. The turnover of labelled protein and release into the medium was followed by determining the amount of GFP, or a normal viral protein, in this case RPV-P, in the cells and medium (Fig. 4). Expressed radiolabelled GFP and RPV-P protein were immunoprecipitated using anti-GFP or anti-RPV-P antibodies, respectively, and the precipitated proteins analysed by SDS–PAGE. GFP was detected at high levels in cells infected with either virus. In each case, the protein detected using anti-GFP antibodies migrated with a molecular mass of approximately 27 kDa, which corresponded to the expected size for GFP. Furthermore, both viruses expressed equivalent quantities of GFP, as similar total levels of GFP were observed in cells infected with either virus. GFP was detected in cell lysates (Fig. 4A) but not in the surrounding medium (Fig. 4B) of RPVINS-GFP-infected cells. The levels of GFP in these cells remained constant throughout the time-course examined, reflecting the stability of the protein. In contrast, GFP was detected both in cell lysates (Fig. 4E) and in

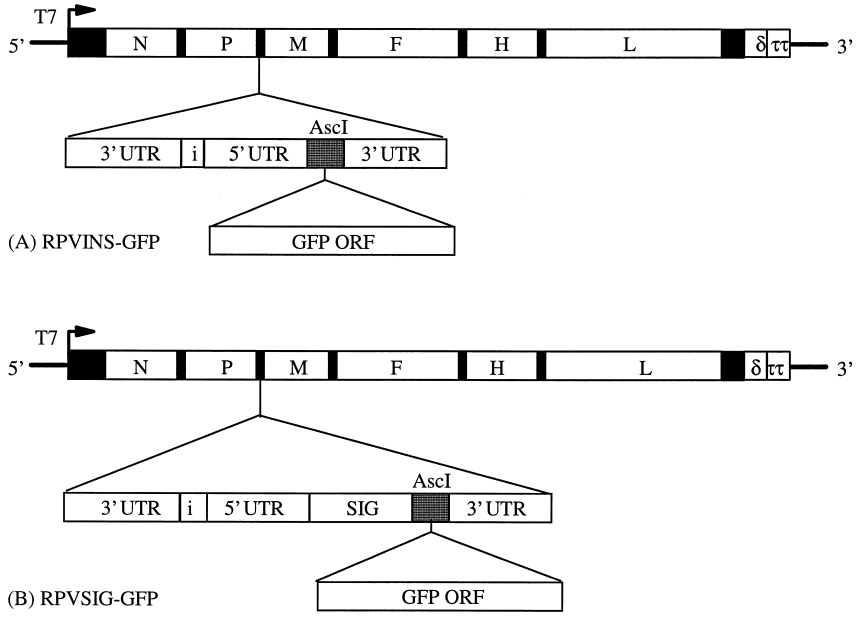

Fig. 1. Diagrams representing the pRPVINS-GFP and pRPVSIG-GFP antigenome cDNA constructs which were made as described in Methods. (A) The pRPVINS-GFP construct was made by inserting the GFP ORF into the unique *Asc*I cloning site in the gene expression cassette, located between the P and M genes of pRPVINS. (B) The pRPVSIG-GFP construct was made by inserting the GFP ORF into the unique *Asc*I cloning site in the gene expression cassette, which includes an N-terminal secretory signal sequence, located between the P and M genes of pRPVSIG. ORFs of RPV are labelled as follows: N, nucleocapsid; P, phosphoprotein; M, matrix; F, fusion; H, haemagglutinin; and, L, large protein of RPV. UTR indicates an untranslated region represented in black in the complete genome diagram, i indicates an intergenic triplet, AscI indicates the unique *Asc*I cloning site, T7 indicates the T7 RNA polymerase promoter, δ indicates the hepatitis delta ribozyme, and $\tau\tau$ indicates the T7 RNA polymerase terminators.

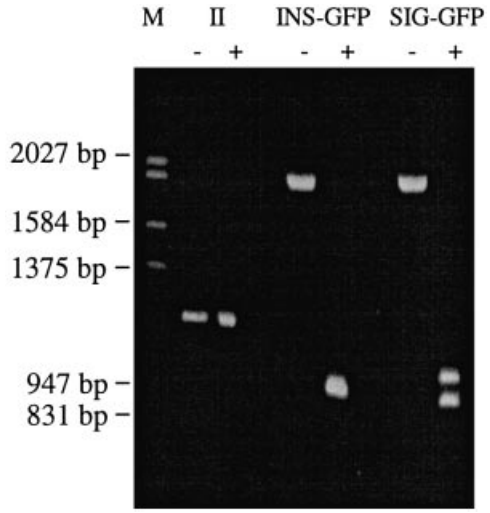

Fig. 2. RT–PCR of viral RNA from RPVII-, RPVINS-GFP- and RPVSIG-GFP-infected cell cultures. PCR was carried out as described in Methods. RT–PCR products for each of the three viruses are shown. The PCR products were digested with *Asc*I to confirm the presence of the restriction site and to distinguish between the two modified recombinant viruses. PCR products were either undigested (−) or digested with *Asc*I (+). II indicates RPVII; INS-GFP indicates RPVINS-GFP; SIG-GFP indicates RPVSIG-GFP.

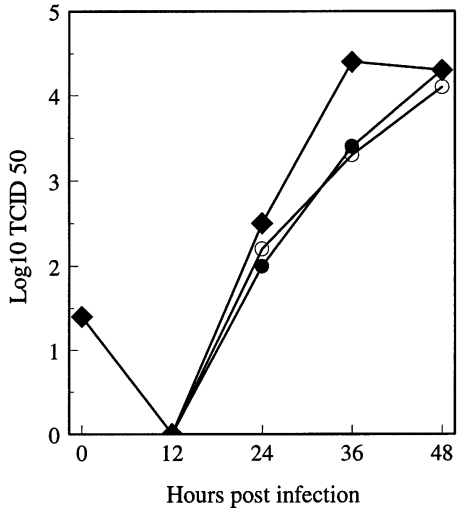

Fig. 3. Comparison of the growth curves of RBOK (◆), RPVINS-GFP (○) and RPVSIG-GFP (●) viruses. Growth rates were examined over a 48 h period in Vero cells.

the medium (Fig. 4F) of RPVSIG-GFP-infected cells. The time-course of the chase showed that GFP was initially found primarily in cells but the level of labelled GFP there decreased with time, whilst the amount of labelled GFP in the medium increased concurrently, showing that the GFP was secreted from infected cells. Therefore, GFP was efficiently secreted from RPVSIG- GFP-infected cells but not from cells infected with RPVINS-GFP.

In the case of both RPVINS-GFP and RPVSIG-GFP viruses, RPV-P protein was detected in cell lysates at constant levels

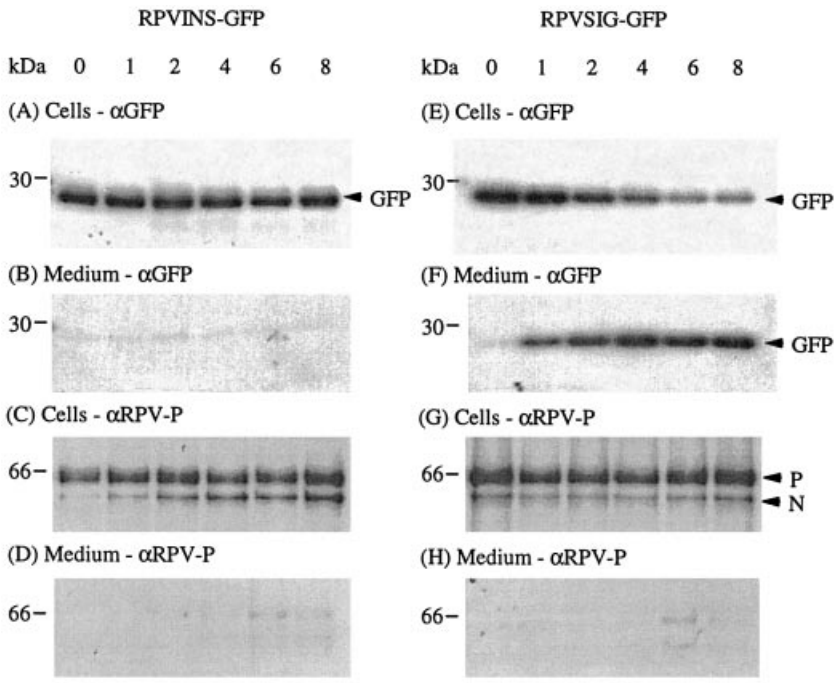

Fig. 4. Analysis of GFP expression and secretion by RPVINS-GFP- and RPVSIG-GFP-virus-infected B95a cells. Radiolabelled proteins from the cell lysate and samples of medium from the pulse–chase experiment were collected at time-points 0, 1, 2, 4, 6 and 8 h, and immunoprecipitated using either anti-GFP or anti-RPV-P antibodies as described in Methods. (A–D) RPVINS-GFP-infected cells and (E–H) RPVSIG-GFP-infected cells showing GFP and RPV-P proteins. Numbers above the gels represent the time in hours after protein labelling at which the samples were collected. GFP indicates green fluorescent protein; P indicates RPV-P protein; N indicates putative RPV-N protein. Positions of molecular mass markers (kDa) are indicated on the left.

for each time-point during the chase period (Fig. 4C, G), but was not detected at any stage in the medium (Fig. 4D, H). The absence of RPV-P protein in the medium of cells infected with either virus and the absence of GFP in the medium of cells infected with RPVINS-GFP confirmed that GFP detected in the medium of RPVSIG-GFP-infected cells was due to active GFP secretion, and was not the result of protein leakage from damaged virus-infected cells. A second protein band which migrated just below the RPV-P band was observed in the RPV-P immunoprecipitation experiment (Fig. 4C, G). This lower band was most likely co-immunoprecipitated with RPV-P protein by anti-RPV-P antibody and corresponded to the position where RPV-N protein would normally be expected (Diallo *et al*., 1987; Baron & Barrett, 1997), suggesting that the N and P proteins are forming N–P complexes (Shaji & Shaila, 1999).

Immunofluorescence of virus-infected cells

We examined the expression and localization of GFP and viral proteins in Vero cells by immunofluorescence (Fig. 5). In cells infected with either virus, GFP fluorescence and anti-GFP antibody immunofluorescence were essentially identical, showing that the fluorescence was solely due to the expressed GFP. Cells infected with either virus always stained positive for both RPV-P and GFP and also showed GFP fluorescence, indicating that GFP was expressed in all virus-infected cells. However, occasionally GFP was observed with only a faint RPV-P signal (Fig. 5D), although this was only found during the early stages after virus infection when low levels of virus-expressed proteins are present. Therefore, at least with the primary and secondary antibodies used in this experiment, antibody-mediated detection of GFP was more sensitive than detection of GFP fluorescence which was more sensitive than antibody-mediated detection of RPV-P protein.

Cells infected with either virus showed separate localization of GFP and RPV-P protein (Fig. 5). The P protein appeared in all cases to be predominantly localized in large cytoplasmic bodies (Fig. 5A, D, G) in what may be virus factories as viral N, L and C proteins colocalize with P in these bodies (M. D. Baron, unpublished observations). In RPVINS-GFP-infected cells, GFP was found throughout the cytoplasm (Fig. 5E, F). In RPVSIG-GFP-infected cells, GFP was observed in a perinuclear and reticular network in the cell cytoplasm (Fig. 5H, I). This indicates that GFP is localized to the endoplasmic reticulum and Golgi complex, the cellular secretory apparatus, which correlates well with the results of the radioimmunoprecipitation experiment. No GFP fluorescence or anti-GFP antibody immunofluorescence was observed in RPVII-infected cells (Fig. 5B, C).

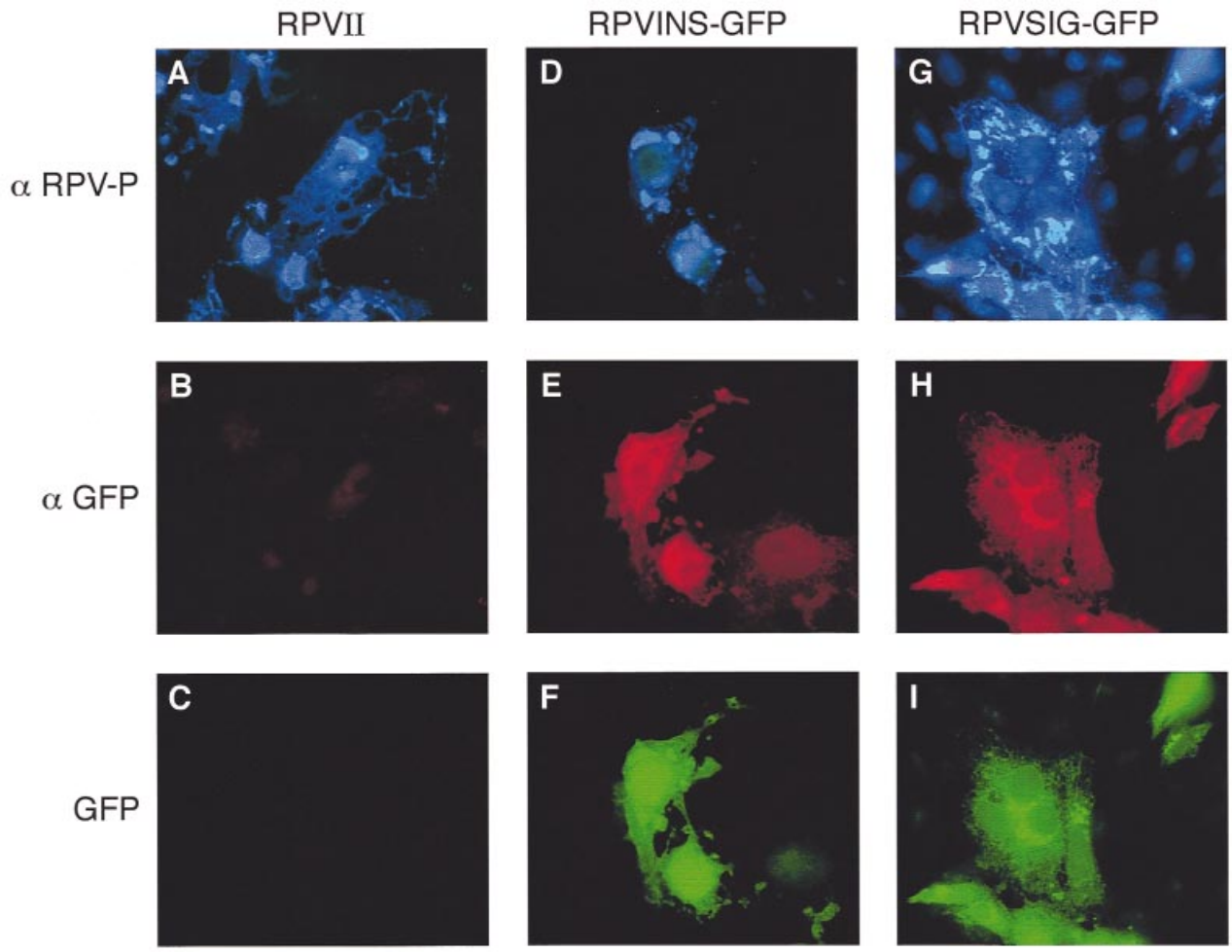

Fig. 5. Expression of GFP and RPV-P proteins in recombinant RPV-infected Vero cells as revealed by immunofluorescence microscopy. (A–C) RPVII-infected cells; (D–F) RPVINS-GFP-infected cells; (G–I) RPVSIG-GFP-infected cells. The relevant virus is indicated on the top and antibody specificity is indicated on the left. GFP fluorescence (GFP) is green, GFP antibody fluorescence (αGFP) is red and RPV-P antibody fluorescence (αRPV-P) is blue. Photomicrograph original magnification 600×.

Animal vaccination tests

To determine the efficacy, immunogenicity and potential pathogenicity of the candidate genetically marked vaccines, they were used in a standard vaccination and challenge experiment. Four cattle (TT32, TT33, TT34, TT35) and two goats (TM34, TM35) were vaccinated with the RPVINS-GFP vaccine, four cattle (TR0, TR1, TQ98, TQ99) and two goats (TM97, TM98) with the RPVSIG-GFP vaccine, and four cattle (TR2, TR3, TR4, TR5) with the control RPVII vaccine. Three to four weeks post-vaccination, the cattle and goats were challenged with the highly virulent RPV-Saudi 1/81 strain or the PPRV-Ivory Coast strain, respectively. No signs of clinical disease associated with rinderpest or PPR infection were observed in any of the animals following either vaccination or challenge. The rectal temperatures in cattle and goats and leukocyte levels in the cattle were monitored as indicators of subclinical disease and viraemia. The rectal temperatures of all the cattle and goats remained within the normal healthy ranges during both vaccination and challenge stages (data not shown). A moderate leukopenia was observed in some of the cattle after vaccination whichever vaccine was used, but leukocyte counts returned to normal levels by 2 weeks post-vaccination (Fig. 6). This mild leukopenia, which is probably indicative of vaccine virus replication, is also commonly observed in cattle vaccinated with the standard RBOK vaccine (T. Barrett, unpublished observations). Signs of mild transient leukopenia were also apparent in some cattle after challenge, whichever vaccine was used, suggesting that replication of the challenge virus had occurred in these animals. A much more dramatic reduction in leukocytes occurs in cattle infected with virulent virus (Anderson *et al.*, 1996).

The cattle and goats were also monitored for the presence of virus or viral RNA in samples of eye secretions and circulating leukocytes. With the exception of a single goat, no virus was detected in the clinical samples from any of the test

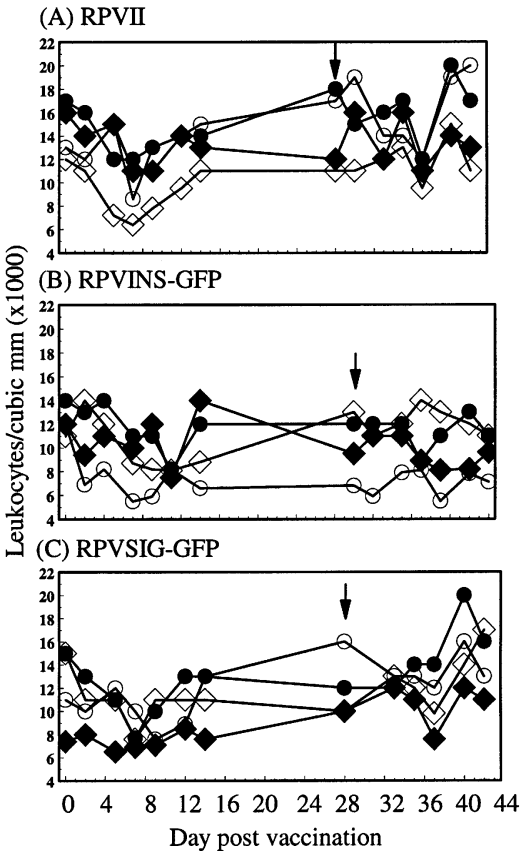

Fig. 6. PBL counts for cattle used in the vaccination and challenge experiment. (A) RPVII (TR2○, TR3◆, TR4◇ and TR5●), (B) RPVINS-GFP (TT32○, TT33◆, TT34◇ and TT35●) and (C) RPVSIG-GFP (TR0○, TR1◆, TQ98◇ and TQ99●). The cattle were vaccinated on day 0 and challenged with the RPV-Saudi 1/81 virus strain on day 28 (RPVII and RPVSIG-GFP) or 30 (RPVINS-GFP). The arrow indicates the day of challenge.

or control vaccinated cattle or goats after either vaccination or challenge. Attempts to isolate virus by cell culture from eye swabs or leukocytes repeatedly proved negative, with the exception of leukocytes but not eye swabs from goat TM97. PPRV was recovered from cell culture of the post-challenge leukocytes of this animal. In addition, no virus RNA was detected either in eye swabs or in circulating leukocytes by RT–PCR with morbillivirus universal PCR primers or RPV-specific PCR primers. Rinderpest RT–PCR positive and negative controls gave the expected results in these tests. Cellular actin mRNA was detected using RT–PCR with bovine actin-specific primers in most RNA preparations, showing that the isolated RNA was of good quality and would enable the detection of RPV-specific RNA present in sufficient quantity in the sample (data not shown).

Antibody responses in vaccinated animals

All 16 vaccinated animals developed high levels of anti-RPV-H antibodies (Fig. 7). Rinderpest humoral antibody responses developed similarly to those of animals vaccinated with the standard cell culture-grown RBOK vaccine. Furthermore, no appreciable anamnestic response was observed upon challenge, suggesting that virus replication was either severely diminished or completely abolished.

The four cattle which had been vaccinated with the RPVII control vaccine, as expected, showed no serum GFP antibody reactivity (Fig. 7A, B, C, D). Similarly, no anti-GFP antibody was detected in the serum of the four cattle and two goats which had been vaccinated with the RPVINS-GFP vaccine even by day 44 post-vaccination (Fig. 7E, F, G, H, M, N). In contrast, GFP antibody was detected in the serum of three of the four cattle and both of the goats which were vaccinated with the RPVSIG-GFP vaccine (Fig. 7I, J, L, O, P). In the case of two of the positive cattle (TR0, TR1) and both goats (TM97, TM98), a high anti-GFP antibody response was detected showing a similar pattern of reactivity to the anti-RPV response. The third positive cow (TQ99) showed a very low anti-GFP antibody response which, over the 49 days, gradually rose to three times above the background level. The anti-GFP response detected in the remaining cow (TQ98) in the RPVSIG-GFP-vaccinated group was not significantly above the prevaccination level even at day 49 post-vaccination.

Discussion

In this report we describe initial experiments to develop a genetically marked recombinant rinderpest vaccine which used GFP as a genetic marker. We produced two modified recombinant RBOK vaccines, RPVINS-GFP and RPVSIG-GFP, which were designed to express intracellular and secreted forms of GFP respectively. A marked vaccine must fulfil several requirements to be of use in the rinderpest eradication campaigns currently under way in Africa and Asia (Rweyemamu & Cheneau, 1995). Genetic modification of the RBOK vaccine must not detrimentally affect the vaccine characteristics of this strain, which includes attenuation, protection from rinderpest and the absence of transmission of vaccine virus to susceptible animals (Plowright & Ferris, 1962; Plowright, 1984). In addition, the marker gene should be stably maintained in the recombinant virus through many virus generations. The vaccine must also produce a strong humoral immune response to the expressed marker protein which can be detected in the serum of all vaccinated animals using an ELISA system.

In the studies reported here, we have shown that the RPVINS-GFP and RPVSIG-GFP viruses possessed all of the characteristics of the standard RBOK vaccine (Plowright & Ferris, 1962). Both vaccines conferred complete protection in cattle against challenge with a highly virulent RPV strain. Following challenge, no clinical signs of rinderpest or any other disease were observed in the cattle. No evidence was found for reversion to virulence for either vaccine which might possibly have resulted from genetic modification of the standard vaccine, which is in accordance with previous work

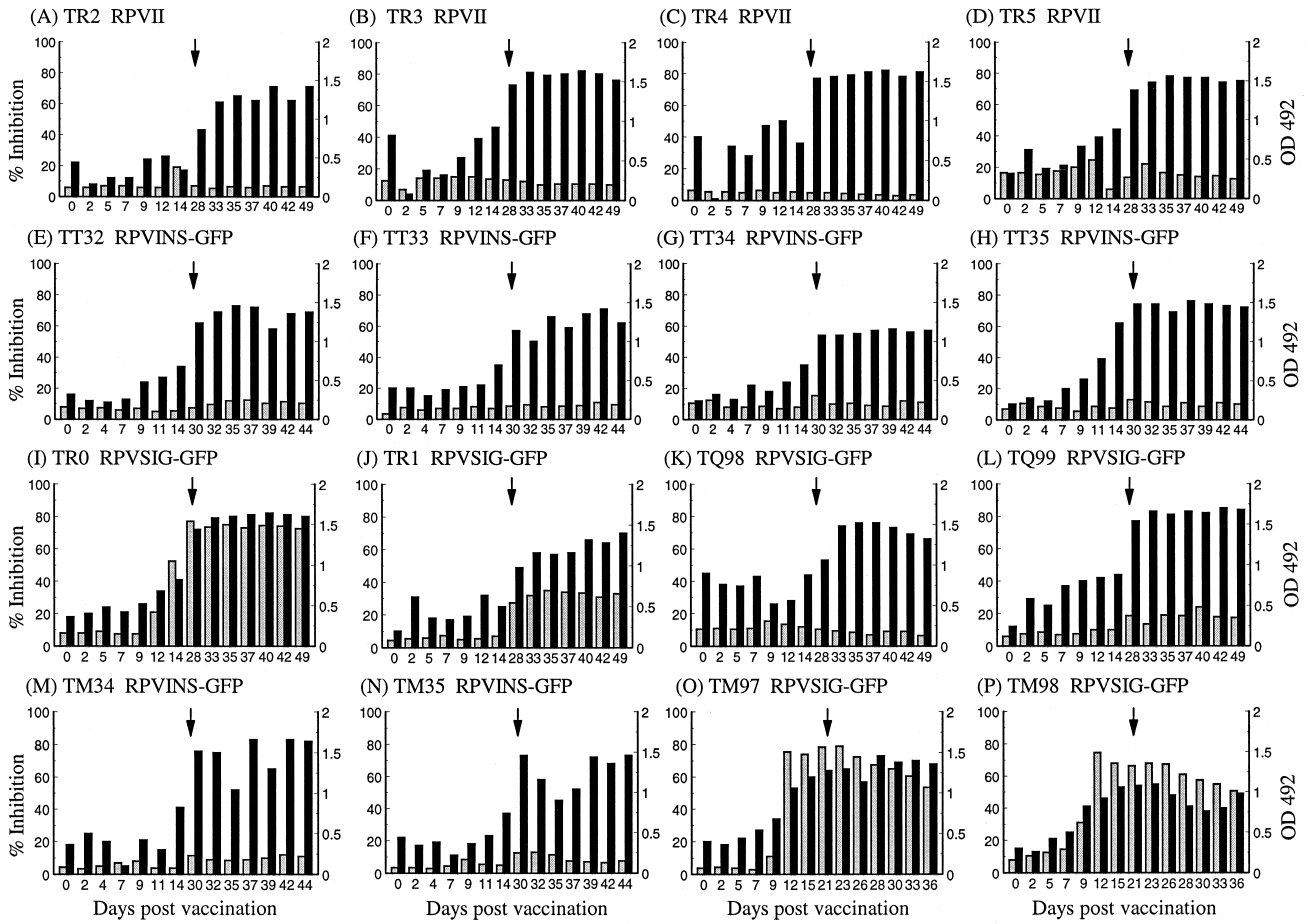

Fig. 7. ELISA detection of anti-RPV-H and anti-GFP antibodies for the cattle and goat vaccination and challenge experiments. (A, B, C, D) RPVII vaccine ELISA results for the four cattle TR2, TR3, TR4 and TR5. (E, F, G, H) RPVINS-GFP vaccine ELISA results for the four cattle TT32, TT33, TT34 and TT35. (I, J, K, L) RPVSIG-GFP vaccine ELISA results for the four cattle TR0, TR1, TQ98 and TQ99. (M, N) RPVINS-GFP vaccine ELISA results for the two goats TM34 and TM35. (O, P) RPVSIG-GFP vaccine ELISA results for the two goats TM97 and TM98. The cattle were vaccinated on day 0 and then challenged with RPV Saudi 1/81 on day 28 for the RPVII and RPVSIG-GFP trial, or day 30 for the RPVINS-GFP trial. The goats were vaccinated on day 0 and then challenged with PPRV Ivory Coast on day 30 for the RPVINS-GFP or day 21 for the RPVSIG-GFP trials. Anti-RPV-H antibody responses are shown by black bars and represent % inhibition shown on the left-hand *x*-axis. Anti-GFP antibody responses are shown by dotted white bars and represent OD_{492} values shown on the right-hand *x*-axis. The arrows indicate the day of challenge in each experiment.

using a recombinant RPV expressing foot-and-mouth disease virus (FMDV) epitopes (Baron *et al.*, 1999). As with the standard cell culture RBOK vaccine, no vaccine or challenge virus-associated viraemia or virus secretion at epithelial surfaces was found for any of the cattle vaccinated with either virus. Both the RPVINS-GFP and RPVSIG-GFP vaccines conferred protection to the vaccinated goats from PPR disease after challenge with a virulent PPRV strain, though post-challenge viraemia was observed in one of the four vaccinated goats. However, this was expected since, although the RBOK vaccine protects goats against severe PPR disease, it does not completely inhibit growth of the heterologous virus (Taylor, 1979; Mariner *et al.*, 1993).

The RPVINS-GFP and RPVSIG-GFP vaccines elicited good anti-rinderpest immune responses in both cattle and goats. Anti-RPV-H antibody responses to both vaccines were similar to those found in animals vaccinated with either the recombinant RPVII or conventional cell culture RBOK vaccines. A marked vaccine should give rise to a strong humoral antibody response to the marker protein in vaccinated animals. It was essential to be able to detect anti-GFP antibodies in the serum of all vaccinated cattle. Our two candidate marker vaccines enabled us to examine the antigenicities of both intracellularly expressed and secreted forms of GFP. RPVINS-GFP failed to give an anti-GFP antibody response in any of the vaccinated cattle or goats. This is in line with experiments using another cytoplasmically expressed protein (FMDV 3D) (Baron *et al.*, 1999). This suggests that in general, cytoplasmic expression is not a viable way to elicit humoral antibody responses, even from proteins with broad spectrum MHC class

II recognition sequences, such as FMDV 3D. Reasoning that we needed to express GFP in such a way as to make it more available to antigen-presenting cells and the MHC class II system, we created a construct in which the GFP would be secreted, thereby making it available to other cells for internalization, degradation and presentation, and also for recognition by specific antibody receptors of B lymphocytes. The success of this approach was shown by five out of six RPVSIG-GFP-vaccinated animals developing anti-GFP antibodies.

However, although three of the four cattle and the two goats vaccinated with RPVSIG-GFP gave rise to anti-GFP antibody responses, one of the cattle (TQ99) showed a low level response, and one (TQ98) was actually completely unresponsive to GFP. Furthermore, although the TQ99 animal's response was positive when compared to the prevaccination anti-GFP levels in our test, it would not be high enough to enable positive and negative sera of vaccinated cattle from field samples to be reliably distinguished using a standardized ELISA. Therefore, secretion of GFP was still insufficient for the generation of a strong anti-GFP antibody response in all vaccinated cattle. There are a number of potential explanations for the variable humoral antibody responses to GFP observed in the RPVSIG-GFP-vaccinated cattle group. Instability of the marker gene in the RPV genome seems unlikely to be the cause for this unresponsiveness. Although we have only grown the recombinant viruses for four passages in cell culture, many similar constructs based on negative-strand viruses have shown that expression from such genes is very stable *in vitro* (Mebatsion *et al*., 1996; He *et al*., 1997; Hasan *et al*., 1997; Singh & Billeter, 1999; Singh *et al*., 1999; Baron *et al*., 1999). We have previously shown that recombinant RPV expressing heterologous viral proteins from the RPVINS gene construct is stable for at least 20 passages (Baron *et al*., 1999). It is therefore probable that expression of GFP will be stably inherited *in vitro* and also *in vivo*. However, it is possible that the foreign gene may be unstable *in vivo*, which might account for the variable antibody responses to the marker protein in different animals, although this question has yet to be experimentally addressed. The variable response may be due to genetic polymorphism in the bovine MHC class II haplotypes. It may be that some animals express MHC molecules which do not efficiently interact with processed GFP peptides. It is possible that GFP does not contain good bovine T-cell or B-cell epitopes, but this seems unlikely since most of the vaccinated animals gave strong anti-GFP responses.

A genetically marked vaccine such as RPVSIG-GFP might be useful as a PPRV vaccine as very good anti-GFP antibody responses were produced in the two RPVSIG-GFP-vaccinated goats, although this will need to be tested in trials using a larger number of goats. At present, RBOK is used to vaccinate goats against PPR in the field. However, as goats can be infected by and transmit rinderpest, vaccination with RBOK has to be discontinued during the rinderpest eradication process (Taylor *et al*., 1995), which would severely hinder the control of PPR disease.

A number of other negative-strand RNA recombinant viruses have been constructed to express foreign proteins as immunizing agents, notably vesicular stomatitis virus (VSV) expressing the influenza virus haemagglutinin (HA) and neuraminidase (NA) glycoproteins (Kretzschmar *et al*., 1997; Roberts *et al*., 1998, 1999), and measles virus expressing the hepatitis B virus surface antigen (Singh *et al*., 1999). In all of these cases a foreign glycoprotein was introduced into the viral envelope. Although this might be a good way of expressing antigen to stimulate the humoral immune response, there are a number of potential risks with this strategy. In particular, expression of receptors for foreign viruses in the viral envelope could potentially give rise to a change in the cell tropism of the recombinant virus. Mainly for this reason, we did not choose a foreign viral surface protein as a marker for our vaccine. We also needed to select a marker protein that cattle or other ruminants would not normally be exposed to in the environment; otherwise serological surveys could give rise to false positive results for unvaccinated animals. For these reasons in particular, GFP appeared to be a good candidate marker protein.

In conclusion, the RPVINS-GFP and RPVSIG-GFP viruses are safe and effective rinderpest vaccines, and RPVSIG-GFP produced good levels of anti-GFP antibodies in most vaccinated animals. However, further studies are required to determine the optimum method for the expression of antigen in our system to produce strong humoral antibody responses to a marker protein in all vaccinated animals. We may actually need to specifically target our marker protein to the cellular endosomal compartments where they can be processed to antigenic peptides and efficiently enter the MHC class II pathway (Marks *et al*., 1995; Boyle *et al*., 1998; Thompson *et al*., 1998). We are therefore constructing recombinant viruses utilizing other genetic markers and alternative marker expression strategies, and will test the efficacy of these vaccines in further trials.

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