Matrix protein and glycoproteins F and H of Peste-des-petits-ruminants virus function better as a homologous complex

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The matrix (M) protein of paramyxoviruses forms an inner coat to the viral envelope and serves as a bridge between the surface glycoproteins (F and H) and the ribonucleoprotein core. Previously, a marker vaccine (RPV-PPRFH) was produced for the control of peste des petits ruminants (PPR) disease, where the F and H genes of Rinderpest virus (RPV) were replaced with the equivalent genes from Peste-des-petits-ruminants virus (PPRV); however, this virus grew poorly in tissue culture. The poor growth of the RPV-PPRFH chimeric virus was thought to be due to non-homologous interaction of the surface glycoproteins with the internal components of the virus, in particular with the M protein. In contrast, replacement of the M gene of RPV with that from PPRV did not have an effect on the viability or replication efficiency of the recombinant virus. Therefore, in an effort to improve the growth of the RPV-PPRFH virus, a triple chimera (RPV-PPRMFH) was made, where the M, F and H genes of RPV were replaced with those from PPRV. As expected, the growth of the triple chimera was improved; it grew to a titre as high as that of the unmodified PPRV, although comparatively lower than that of the parental RPV virus. Goats infected with the triple chimera showed no adverse reaction and were protected from subsequent challenge with wild-type PPRV. The neutralizing-antibody titre on the day of challenge was ~17 times higher than that in the RPV-PPRFH group, indicating RPV-PPRMFH as a promising marker-vaccine candidate.

INTRODUCTION

Morbilliviruses cause clinically important diseases, such as human measles, and economically devastating diseases of domestic and wild animals, including distemper, cattle plague (rinderpest) and sheep and goat plague (peste des petits ruminants) (Barrett & Rossiter, 1999). They belong to the genus Morbillivirus in the family Paramyxoviridae and form a small group of antigenically related viruses. The virions are pleomorphic particles with a lipid envelope enclosing a ribonucleoprotein core that contains the genome, a single strand of RNA of negative polarity, which is encapsidated by the nucleocapsid (N) protein. The morbillivirus genome is just under 16 kb in length and is organized into six contiguous, non-overlapping transcription units separated by short intergenic regions and encoding six structural proteins (N, P, M, F, H and L) in the order 3′-N-P(C/V)-M-F-H-L-5′ (Bailey et al., 2005; Crowley et al., 1988). In addition, there are two non-structural proteins (C and V), which are translated from the P gene open reading frame (ORF) by different mechanisms (Bellini et al., 1985; Cattaneo et al., 1989; Mahapatra et al., 2003).

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease that is often fatal in small ruminants. It is now widespread in parts of west and sub-Saharan Africa, the Middle East and on the Indian subcontinent. Because of the strong antigenic relationship among the morbilliviruses, PPR disease has been controlled for many years by the use of a rinderpest virus (RPV) tissue culture-adapted vaccine (Plowright & Ferris, 1962). Due to the ongoing global rinderpest-eradication programme, the RPV vaccine can no longer be used in any species within rinderpest-free zones to ensure a serologically negative population. A homologous peste-des-petits-ruminants virus (PPRV) vaccine has been produced by passaging the Nigeria 75/1 strain of PPRV 63 times in Vero cells to attenuate it fully (Diallo et al., 1989) and this vaccine is now being introduced into some PPR-endemic regions in Africa and the Middle East. However, a major drawback of the currently used attenuated morbillivirus vaccines is that the vaccinated animals develop a full range of immune responses to viral proteins and therefore these animals cannot be distinguished serologically from those that have recovered from natural infection. This causes difficulties in disease surveillance (Anderson & McKay, 1994), as the sera from both vaccinated and naturally infected animals produce similar results in standard serological tests. One way to

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overcome this difficulty would be to use marker vaccines, i.e. vaccines that allow serological identification of the vaccinated animals, in place of the currently used attenuated tissue-culture vaccines. To this end, Das et al. (2000a) produced a chimeric RPV vaccine bearing the surface glycoproteins of PPRV (RPV-PPRFH virus), but this was found to grow poorly in tissue culture. The poor growth of the RPV-PPRFH chimeric virus was postulated to be due to inefficient budding of the chimeric virus from the host cell and thus could have resulted from incompatibility of the interaction of the surface glycoproteins with the internal components of the virus, in particular with the (M) protein. The M protein lies beneath the virion envelope and interacts with the internal nucleocapsid and the cytoplasmic tails of the surface glycoproteins. It is believed to play a very significant role in morbillivirus assembly and budding by concentrating the F and H proteins, as well as the ribonucleocapsid, at the virus-assembly site (Cathomen et al., 1998a; Peeples, 1991). It was hoped that the growth characteristics of this virus could be improved by incorporating the M protein component from PPRV. In the present study, we have reported the rescue and characterization of two chimeric RPVs in which the M protein gene (RPV-PRPM) or the genes encoding the M, F and H proteins (RPV-PRPMFH) of RPV were replaced with those from PPRV.

METHODS

Cells and viruses. Vero cells were used to grow PPRV and RPV for RNA isolation and also for rescue and growth of the chimeric viruses. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 25 mM HEPES (pH 7.2) with 5% fetal calf serum and penicillin (100 U ml⁻¹) and streptomycin (100 mg ml⁻¹).

The rescued recombinant virus RPV2C or the conventional PPRV vaccine (Nigeria 75/1) (Diaglo et al., 1989) was used to inoculate Vero cells. When the cytopathic effect (CPE) was almost complete, virus was prepared by a single freeze-thaw cycle, followed by removal of cell debris by centrifugation at 1280 g for 10 min. The titre of both viruses was measured by estimating the TCID₅₀ on Vero cells. The rescued recombinant viruses were grown and titrated on Vero cells as described previously. Recombinant fowlpox virus (FP-T7) was grown in primary chick embryo fibroblasts as described previously (Das et al., 2000a).

Plasmids and molecular-biology techniques. All DNA manipulations and cloning were carried out by using standard protocols. The plasmids pKS-N, pKS-P, pGEM-L, pRPV2C and pRPV-PPRFH have been described elsewhere (Baron & Barrett, 2000; Das et al., 2000a). RNA extraction from cultures, purified peripheral blood leukocytes (PBLs) and eye swabs was carried out by using Trizol (Invitrogen) as described by Das et al. (2000a). RT-PCR using Taq polymerase for analytical purposes and Pfu polymerase for preparative purposes was performed as described by Forsyth & Barrett (1995) and Baron et al. (1999), respectively.

Cloning of the M gene of PPRV. In order to manipulate the M gene, restriction sites for SbfI (at the end of the P gene) and SfiI (immediately after the M gene ORF) of the plasmid pRPV2C were used. The same two restriction sites were incorporated into the PPRV M gene copy. The upstream primer PPRSBF (5'-CCGCGCCGCGCAGCTCGGTGAAACACAYCCCTCT-3'), nt 1606–1637, SbfI site underlined) containing the SbfI restriction site at the end of PPRV P gene was designed by using published PPRV P gene sequence data (Mahapatra et al., 2003). Similarly, published sequence data for the PPRV M gene (Haffar et al., 1999) were used to design the downstream primer PPRSSWA (5'-GCAGTGTTAATATCTAGGTTAAGTCTGTT-3'), nt 1062–1029, SfiI site underlined) containing the SfiI restriction site immediately downstream of the M gene ORF. The M gene ORF was amplified by using RNA from Vero cells infected with the PPRV vaccine strain and the 1100 bp amplified product was cloned into the pGEM5Zf vector. A clone containing the M gene ORF of PPRV (pPMPRM) was sequenced completely on both strands and the sequence was compared with the published sequence to ensure that there were no PCR-induced mutations.

Construction of genome plasmids. The SbfI/SfiI digestion product of plasmid pPMPRM was used to replace the M gene ORF of plasmid pRPV2C to make the full-length genome pRPV-PPRM. For construction of the plasmid pRPV-PPRMFH, a different strategy was followed. As the plasmid pRPV-PPRFH was based on the pRPV2B vector, which lacks the SbfI site at the end of the P gene, SbfI and SfiI restriction sites could not be used for the M gene swap. Instead, the restriction sites for ClaI, present at the beginning of the N gene (nt 213), and NotI, present downstream of the M gene ORF (nt 4814), were used for the construction of this plasmid. The NotI–ClaI digestion product of plasmid pRPV-PPRM was ligated with the ClaI–NotI digestion product of the plasmid pRPV-PPRFH to make the full-length genome plasmid pRPV-PPRMFH. Restriction-enzyme analysis was carried out to confirm that the plasmids contained full-length copies of the viral genome, and the M genes of both constructs were sequenced to ensure that they were from the desired virus.

Transfection and recovery of infectious recombinant viruses. Vero cells in six-well plates were transfected as described previously (Das et al., 2000b) with slight modifications. Briefly, cells at ~70% confluence were infected with FP-T7 virus at an m.o.i. of 0.1 for 1 h and then transfected with pKS-N, pKS-P, pGEM-L and the appropriate genome plasmid by using TransFast (Promega) as the transfecting reagent, following the manufacturer’s instructions. Serum-free medium (DMEM) was used to prepare the DNA/TransFast mix and to wash the cells. Transfected cells were observed daily under the microscope for the appearance of signs of virus-infected CPE. Cells were trypsinized 4–5 days post-transfection, transferred to a 75 cm² flask and grown until the development of CPE.

Virus characterization. In order to characterize the chimeric viruses, RT-PCR was carried out on total RNA isolated from virus-infected Vero cells. The primers used were UPP-F (5'-ATGGTTTATGATCACACCCGTTG-3', morbillivirus P gene, nt 390–410) and M2R (5'-GTATCGACCCCGCTGCT-3', PPRV M gene, nt 130–112) for the M gene, and PPRV F gene-specific primers F1b (5'-AGTCAAAAGATTCTGATCACAGT-3', nt 760–784) and F2d (5'-GGGTCTCGAAGGCTAGGCCCGTG-3', nt 1207–1183). Multi-step growth curves and examination of the plaque morphology of parental and recombinant viruses were carried out as described previously (Das et al., 2000a).

Confocal fluorescence microscopy. Vero cells infected with recombinant viruses were grown in 25 cm² flasks. At 24 h post-infection, cells were trypsinized and plated on coverslips in six-well plates (3 × 10⁵ cells per well). After 48 h, cells were fixed by using 3% paraformaldehyde for 20 min followed by three washes with Ca²⁺/Mg²⁺-free PBS. The background fluorescence of the cells was quenched with 50 mM NH₄Cl, followed by three washes with Ca²⁺/Mg²⁺-free PBS. Cells were then permeabilized by treatment with 0.1% Triton X-100 (Sigma) in Ca²⁺/Mg²⁺-free PBS for 5 min, followed by three washes in Ca²⁺/Mg²⁺-free PBS. Non-specific binding of antibodies to cells was blocked by incubating the cells for
5 min in Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS containing 0-2% gelatin. Cells were then labelled for surface or internal proteins by using CV7 or F122 monoclonal antibody (mAb) as required. F122 (a kind gift from M. Sugiyama, Gifu University, Japan) and CV7 (provided by W. J. Bellini, CDC, Atlanta, GA, USA) are mAbs raised against RPV F protein and measles virus M protein, respectively. The secondary antibody used was Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes). Cells were then stained with 4,6-diamidino-2-phenylindole (DAPI; diluted 1:10 000) for 10 min for nuclear staining. Coverslips were mounted with Vectashield (Vector Laboratories) and observed under a confocal microscope (Leica TCS SP2).

**Animal studies.** Outbred, indigenous British white goats of 6–12 months of age were used for the vaccination trial, which was carried out under Biosafety Level 2 with regard to staff and at Level 4 with regard to escape of the pathogen into the environment. Goats were housed in the isolation facility of the Institute for Animal Health and observed for 4 weeks in the isolation unit prior to the beginning of the experiment, to ensure that they were in good health. Stocks of vaccine virus were grown on Vero cells; the challenge virus has been described elsewhere (Das et al., 2000a). In this experiment, three goats (UQ51–UQ53) were vaccinated with the conventional PPR vaccine, four (UQ54–UQ57) with RPV-PPRPMFH virus, three (UQ58–UQ60) with RPV-PPRFH virus and three (UQ61–UQ63) were kept as unvaccinated controls. Vaccine virus (10\(^{5.5}\) TCID\(_{50}\)) was injected into the animals in the shoulder region as a single, subcutaneous dose. All animals were challenged with virulent PPRV (Ivory Coast 89/1) 4 weeks after vaccination. The animals were examined daily for the appearance of clinical signs of PPR disease. Rectal temperatures and total leukocyte counts were monitored for 2 weeks following vaccination and challenge. Clinical samples were collected and analysed as described previously (Das et al., 2000a). Virus isolation from PBLs was attempted by co-cultivation with Vero cells. In order to detect the viral RNA in clinical samples (PBLs and eye swabs), a simple diagnostic PCR was carried out with the primer set F1b/F2d, specific for the PPRV F gene. A nested PCR using the primer set F1 (5\'-ATACACAGTTAAGGCTTAGG-3', PPRV F gene, nt 777–801) and F2 (5\'-GAGACTGAGTTTGTGACCTACA-3', PPRV F gene, nt 1148–1124) was also carried out to enhance the sensitivity of detection of the viral RNA; this nested PCR was carried out only on negative samples obtained from the PCR using the diagnostic primer set F1b/F2d.

The virus-specific antibody response was determined by using the procedure described by Anderson et al. (1996). This assay determines the amount of antibody in a serum sample that recognizes a specific viral antigen by the ability of that sample to inhibit the binding of an antigen-specific mAb to viral antigen. The results were expressed as percentage inhibition of binding of the control mAb. The cut-off value between negative and positive serum was taken as 50% inhibition. Tests for PPRV-neutralizing antibodies were carried out on microtitre plates following the method described by the OIE (2000).

**RESULTS**

**Recovery of recombinant viruses from cloned cDNA**

In order to rescue chimeric virus from the cloned cDNA, Vero cells were transfected with full-length genome plasmids of either pRPV-PPRM or pRPV-PPRPMFH (Fig. 1) along with the helper N, P and L plasmids. In the case of the RPV-PPRM virus, CPE appeared 3–4 days after transfection, at the same time as the control RPV plasmid pRPV2C, whereas RPV-PPRPMFH virus required one further passage for the development of CPE. Two independent cDNA clones of RPV-PPRM virus and three independent clones of RPV-PPRPMFH virus were rescued. However, only one clone in each case was used for further characterization. In order to confirm the identity of the recombinant viruses, RT-PCR was carried out on the RNA extracted from infected Vero cells using the primer pair UPP-F/M2R, which produced a fragment of the expected size of ~1400 bp (data not shown). Similarly, the PPRV F gene-specific primer set F1b and F2d was used in the case of RPV-PPRPMFH virus, which produced an amplified product of the expected size of ~450 bp (data not shown). No PCR products were generated in parallel reactions in which reverse transcriptase was omitted, indicating that the amplified products were not generated from the transfected plasmid DNA. The PCR products were sequenced to ensure that they were from the expected virus.

**Growth of chimeric viruses in tissue culture**

Standard multi-step growth curves were carried out to compare the growth of the recombinant viruses with that of the parental viruses. RPV-PPRM virus grew to the same titre as the parental RPV2C (Fig. 2a), indicating that replacing the RPV M gene with that from PPRV had no apparent adverse effect on growth rate of the chimeric virus. RPV-PPRPMFH virus had an initially slow growth rate, but the final titre was almost as high as that of the non-recombinant PPRV in Vero cells (Fig. 2b), although it was comparatively...
lower than that of RPV. The poorest-growing virus was RPV-PPRFH, achieving a titre similar to that reported by Das et al. (2000a). This experiment was repeated with similar results.

**Plaque morphology**

Vero cells infected with the parental or chimeric viruses were overlaid with carboxymethylcellulose and the plaques were stained with Giemsa. Both parental viruses, RPV and PPRV, produced small syncytia (Fig. 3a, b, respectively). Phenotypically, RPV-PPRM virus was found to be similar to the parental viruses (Fig. 3c). In the case of RPV-PPRMFH virus, however, very large syncytia were observed (Fig. 3d), which was found to be the same phenotype as that produced by the RPV-PPRFH virus (Fig. 3e).

**Localization of viral proteins by confocal microscopy**

In order to study the effect of replacement of the RPV M protein with that from the related virus PPRV on the distribution pattern of viral proteins, virus-infected cells were examined by confocal microscopy after labelling with an anti-F or anti-M mAb. As illustrated in Fig. 4, the distribution pattern of the M and F proteins in RPV-PPRM-infected cells was found to be the same as that of the parental virus, i.e. M protein was clearly observed at the cell periphery, whilst the F protein was located on the cell surface and also inside the cell (Fig. 4a, b). In the case of the RPV-PPRFH and RPV-PPRMFH viruses, giant, multi-nucleated cells were generally found and it was difficult to locate a non-syncytial infected cell. In RPV-PPRMFH-infected cells, both the F and M proteins were clearly visible at the cell periphery (Fig. 4c, d), whereas in RPV-PPRFH-infected cells, the F protein was mostly found inside the cell in bright patches, whilst the M protein was clearly distributed at the periphery of the cells (Fig. 4e, f).

**In vivo characterization of RPV-PPRMFH virus**

A preliminary vaccination trial was carried out to study the efficacy of the triple-chimera RPV-PPRMFH virus as a potential marker vaccine in goats by comparing it with the parental PPRV vaccine and the double-chimera vaccine, RPV-PPRFH. No specific clinical signs of PPR disease were observed following vaccination in any of the animals used in these experiments. The temperature of all of the vaccinated animals remained within the normal range (data not shown). In the majority of vaccinated animals, there was an initial leukocytosis on day 2 followed by a slight decline in leukocyte count up to day 9 (data not shown), but the count always remained within the normal range for goats. Following challenge, none of the vaccinated animals showed any constant rise in body temperature or leukopenia (data not shown) or exhibited any PPR-specific clinical signs, except for a watery nasal discharge in some animals in the RPV-PPRMFH- and RPV-PPRFH-vaccinated groups on days 2 and 3 following challenge. In contrast, the unvaccinated control animals showed pyrexia, leukopenia and PPR-specific clinical signs following challenge. Animal UQ63 showed the most severe clinical disease and had to be euthanized on day 7; a post-mortem was carried out, which revealed mild pathology. Clinical signs of infection in the eyes and oral/nasal mucosae were observed up to day 11 following challenge in the control animals, after which they recovered from the infection, following the normal pattern of disease in British goats.

**Detection of virus and viral RNA in clinical samples**

Attempts were made to isolate virus from PBLs of vaccinated and control animals. Virus could be isolated on day 5 from some animals vaccinated with chimeric viruses (Table 1). This was in agreement with our observation of the presence of viral RNA in lymphocytes on day 5, as evidenced by RT-PCR (Table 2). Viral RNA was detected in ocular swabs and lymphocytes mainly on days 2 and 5 following vaccination, after which levels declined and it could only be detected by nested PCR (Table 2). Tests to detect the presence of viral RNA in ocular swabs and lymphocytes were not carried out on the animals that were vaccinated with tissue culture-attenuated PPR vaccine. Following challenge, there was evidence of replication of challenge virus in the case of vaccinated animals; this was not sufficient to be detected by
Fig. 3. Plaque morphology of the parental and chimeric viruses. Vero cells were infected with ~500 TCID₅₀ of each virus and cultured under carboxymethylcellulose. Four days after infection, cells were stained with Giemsa and photographed by using a digital camera (Canon Digital D60 with 65 mm macro lens). Bars, 0.5 μm.

Fig. 4. Confocal microscopy of cells infected with the chimeric viruses. Vero cells plated on coverslips in six-well plates (3 × 10⁵ cells per well) were infected with 600 TCID₅₀ of the chimeric virus. After 36–40 h, cells were fixed and stained with either F122 (a, c, e) or CV7 (b, d, f) mAb, followed by Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence was observed by using a Leica confocal microscope. Bars, 15 μm.
virus isolation or even by a simple diagnostic PCR, but could be detected by the more sensitive nested PCR on days 2–9 (Table 2, columns 4 and 6). In the unvaccinated control group, viral RNA was detected in PBLs on days 2 and 5 following challenge in all animals and in some up to day 7 (Table 2, columns 7 and 8).

**Antibody detection by competitive ELISA**

Competitive ELISA was carried out on serum collected from the vaccinated and challenged animals to detect the antibody response using RPV and PPRV H-specific mAbs as described in Methods. None of the animals used in the experiments showed an antibody response to either RPV or PPRV on the day of vaccination (Fig. 5). All of the animals vaccinated with PPRV or RPV-PPRMFH showed >50% inhibition of PPRV H-specific mAb binding on the day of challenge (Fig. 5a and b). In contrast, none of the animals vaccinated with RPV-PPRFH showed any PPRV H-specific inhibition on the day of challenge, but a response to PPRV was observed following challenge (Fig. 5c). The unvaccinated control animals also showed PPR H-specific inhibition following challenge (Fig. 5d). None of the animals used in the experiments showed an RPV H-specific inhibition >28%, a level considered to be non-specific in the test, and it was always lower than the PPRV H-specific inhibition.

**Table 1. Virus isolation from PBLs**

PBLs from each animal were co-cultivated with Vero cells following vaccination and challenge. Numbers in parentheses are values post-challenge. NT, Not tested.

<table>
<thead>
<tr>
<th>Time post-challenge</th>
<th>No. animals positive for virus isolation/total no. tested</th>
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<tbody>
<tr>
<td></td>
<td>PPRV</td>
</tr>
<tr>
<td>Day 0</td>
<td>0/3 (0/3)</td>
</tr>
<tr>
<td>Day 2</td>
<td>1/3 (0/3)</td>
</tr>
<tr>
<td>Day 5</td>
<td>3/3 (0/3)</td>
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<tr>
<td>Day 7</td>
<td>1/3 (0/3)</td>
</tr>
<tr>
<td>Day 9</td>
<td>1/3 (0/3)</td>
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<tr>
<td>Day 12</td>
<td>0/3 (0/3)</td>
</tr>
<tr>
<td>Day 14</td>
<td>0/3 (0/3)</td>
</tr>
</tbody>
</table>

**Table 2. Detection of viral RNA by RT-PCR**

RNA was extracted from eye swabs (E) or PBLs and subjected to RT-PCR as described in Methods. The PCR product obtained by using the diagnostic primer set F1b/F2d was used as template for the nested PCR (primer set F1/F2). Eye swabs were collected only up to day 7 following vaccination. Data in parentheses indicate values post-challenge. NT, Not tested.

<table>
<thead>
<tr>
<th>Time post-challenge</th>
<th>Sample</th>
<th>No. animals positive for the presence of viral RNA/total no. tested</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>RPV-PPRMFH</td>
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<tr>
<td></td>
<td></td>
<td>F1b/F2d</td>
</tr>
<tr>
<td>Day 0</td>
<td>E</td>
<td>0/4</td>
</tr>
<tr>
<td>Day 2</td>
<td>PBLs</td>
<td>0/4 (0/4)</td>
</tr>
<tr>
<td>Day 5</td>
<td>E</td>
<td>4/4</td>
</tr>
<tr>
<td>Day 7</td>
<td>PBLs</td>
<td>4/4 (1/4)</td>
</tr>
<tr>
<td>Day 9</td>
<td>E</td>
<td>4/4</td>
</tr>
<tr>
<td>Day 12</td>
<td>PBLs</td>
<td>4/4 (0/4)</td>
</tr>
<tr>
<td>Day 14</td>
<td>E</td>
<td>0/4</td>
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<tr>
<td></td>
<td>PBLs</td>
<td>1/4 (1/4)</td>
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<tr>
<td></td>
<td>E</td>
<td>NT</td>
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<tr>
<td></td>
<td>PBLs</td>
<td>0/4 (0/4)</td>
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<tr>
<td></td>
<td>E</td>
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<td></td>
<td>PBLs</td>
<td>0/4 (0/4)</td>
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<td></td>
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<td></td>
<td>PBLs</td>
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Neutralizing-antibody titres

Serum neutralization assays were carried out to determine the virus-neutralizing antibody titres, if any, to PPRV in the serum of vaccinated and control animals before and after challenge with virulent PPRV. As shown in Table 3, on day 0, all of the animals were negative for the presence of PPRV-specific neutralizing antibody and all of the vaccinated animals had significant neutralizing-antibody titres on the day of challenge. The neutralizing-antibody titre of the RPV-PPRMFH group was found to be as high as that of the PPRV group and >17 times higher than the RPV-PPRFH group. No neutralizing antibody was detected in any of the control animals on the day of challenge (Table 3).

DISCUSSION

Substitution of the RPV M protein with that of PPRV did not have any adverse effect on growth of the virus in tissue culture, reflecting the high level of M protein conservation among morbilliviruses. The M proteins of RPV and PPRV are 92.5% similar and 85% identical at the amino acid level and this high degree of conservation reflects the fact that the M protein plays a highly important role in the formation of new virus particles by interacting with the surface glycoproteins in the cell membrane and with the internal nucleocapsid structures in the cytoplasm. When grown on Vero cells, the RPV-PPRM virus was found to be similar phenotypically to the parental RPV. The cellular distribution of the viral F and M proteins was also the same as in cells infected with RPV. In this virus, there was non-homologous interaction between the N and M proteins and also between the M and the surface glycoproteins. However, these interactions appeared to be equivalent and the non-homologous M apparently did not have a measurable effect on the replication efficiency of the recombinant virus, at least in Vero cells.

Previously, our group has recovered virus from cDNA copies of the RPV genome in which the F and H genes were replaced by the corresponding genes from PPRV; however, the virus was found to be highly debilitated in tissue culture.
reduce the fusion and increase the replication efficiency of the chimera by incorporating the homologous PPRV M protein.

The chimeric RPV-PPRMFH virus was produced with the aim of rectifying the poor growth of the RPV-PPRFH chimera by incorporating the homologous PPRV M protein. It was expected that the homologous M protein would reduce the fusion and increase the replication efficiency of the chimeric virus. However, although the virus did grow to a higher titre than RPV-PPRFH virus, its growth rate was initially slower than and the final titre was not as high as that of the parental RPV and large syncytia were still observed in virus-infected cells. In other paramyxoviruses, the M protein has been shown to be responsible for the incorporation of nucleocapsids into virions (Coronel et al., 2001; Sakaguchi et al., 2002). In the RPV-PPRMFH virus, there is homologous interaction between the M protein and the glycoprotein tails, but the interaction between the M protein and the nucleocapsid is non-homologous. The poor growth and large-syncytium-forming phenotype of the chimera compared with RPV cannot be due to the non-homologous interaction between the PPR M protein and the RPV nucleocapsid, as in the case of RPV-PPRM virus, this was not a problem. However, it may be that the interaction of the PPRV surface glycoproteins with the M protein are not the same as those of the RPV glycoproteins with the M protein, leading to a significantly greater specificity of PPRV F and H for their homologous M protein. It is also possible that there are other differences between these viruses, e.g. in the efficiency of folding of the glycoproteins, which would mean that no chimera with PPRV glycoproteins could be as growth-competent as RPV (our PPRV isolate did not grow as well as RPV in cell culture). Further chimeras based on PPRV will be required to resolve this issue; however, in the absence of a reverse-genetics system for PPRV, these experiments could not be carried out. Research is ongoing to establish a reverse-genetics system for PPRV, which may provide an opportunity to continue this work.

A preliminary vaccination trial was conducted to evaluate the triple-chimera RPV-PPRMFH virus as a marker vaccine and also to compare its efficacy with that of the tissue culture-attenuated PPRV and the double chimera, RPV-PPRFH. Both the vaccinated and control animals were housed in the same isolation unit throughout the period of study. Although the vaccine virus was detected in ocular secretions and was possibly present in other secretions and excretions, none of the control animals showed evidence of infection with the vaccine and were negative for neutralizing antibodies on the day of challenge, indicating that the vaccine virus does not spread by contact, despite close interaction and communal water and food supplies. The absence of clinical responses, including fever and leukopenia, showed that these vaccines are safe to use, at least in goats.

The competitive ELISA based on response to the H protein showed that all of the animals vaccinated with chimeric vaccines were positive for PPRV–specific inhibition, whereas they remained negative for RPV–specific inhibition. Thus, the mAb tests based on the response to the H (Anderson & McKay, 1994) and N (Libeau et al., 1992, 1995) proteins of RPV and PPRV could be used to distinguish between vaccinated and naturally recovered animals and also vaccinated animals that subsequently became infected (Barrett et al., 2003). Neutralizing antibodies were detected in all of the vaccinated animals. Whilst the RPV-PPRMFH- and

### Table 3. Serum neutralizing-antibody responses to PPRV in goats

Results are given as the log_{10} titre that gave 50% neutralization. –, Negative.

<table>
<thead>
<tr>
<th>Virus/goat no.</th>
<th>Vaccination (day 0)</th>
<th>Challenge (day 0)</th>
<th>Two weeks post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPRV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UQ51</td>
<td>–</td>
<td>≥3·51</td>
<td>4·11</td>
</tr>
<tr>
<td>UQ52</td>
<td>–</td>
<td>≥3·58</td>
<td>4·35</td>
</tr>
<tr>
<td>UQ53</td>
<td>–</td>
<td>≥3·51</td>
<td>4·26</td>
</tr>
<tr>
<td>RPV-PPRMFH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UQ54</td>
<td>–</td>
<td>≥3·41</td>
<td>4·18</td>
</tr>
<tr>
<td>UQ55</td>
<td>–</td>
<td>≥3·24</td>
<td>≥4·48</td>
</tr>
<tr>
<td>UQ56</td>
<td>–</td>
<td>≥3·33</td>
<td>≥4·41</td>
</tr>
<tr>
<td>UQ57</td>
<td>–</td>
<td>3·26</td>
<td>4·03</td>
</tr>
<tr>
<td>RPV-PPRFH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UQ58</td>
<td>–</td>
<td>1·75</td>
<td>≥4·56</td>
</tr>
<tr>
<td>UQ59</td>
<td>–</td>
<td>2·43</td>
<td>≥4·56</td>
</tr>
<tr>
<td>UQ60</td>
<td>–</td>
<td>2·05</td>
<td>≥4·48</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UQ61</td>
<td>–</td>
<td>–</td>
<td>≥4·33</td>
</tr>
<tr>
<td>UQ62</td>
<td>–</td>
<td>–</td>
<td>≥4·26</td>
</tr>
<tr>
<td>UQ63</td>
<td>–</td>
<td>–</td>
<td>Dead</td>
</tr>
</tbody>
</table>

and produced very large syncytia (Das et al., 2000a). The defective growth of RPV-PPRFH was thought to be the result of inefficient interaction of the cytoplasmic domains of one or both of the PPRV glycoproteins with the RPV M protein. The cellular distribution of the F and M proteins in all of the recombinant chimeric RPV-PPRVs was analysed. In the case of RPV-PPRFH virus, the F protein was found inside the cell in discrete patches, unlike the other viruses where the F protein was observed mostly on the cell surface. This could be due to the defective interaction of the F and M proteins, resulting in altered distribution or transport of the F protein in the infected cells. Previously, Cathomen and colleagues reported that, in cells infected with a measles virus mutant where the M protein gene had been deleted, the ribonucleocapsid and glycoproteins largely lost co-localization, confirming the role of M protein as the virus-assembly organizer. The M-deficient measles virus mutant was found to be considerably more efficient in inducing cell–cell fusion and the virus yield was also reduced dramatically (Cathomen et al., 1998b; Mebatison et al., 1999). It was suggested by these authors that the association of M with the cytoplasmic tails of the glycoproteins negatively influenced their fusion efficiency (Cathomen et al., 1998a, b) and this may also be a plausible explanation for the high fusogenic activity of RPV-PPRFH virus.

The chimeric virus does not spread by contact, despite close interaction and communal water and food supplies. The absence of clinical responses, including fever and leukopenia, showed that these vaccines are safe to use, at least in goats.
PPRV-vaccinated groups had higher neutralizing-antibody titres than the RPV-PPRFH-vaccinated group on the day of challenge, all of the animals were protected from subsequent challenge, indicating that these vaccines are efficacious and can be used as genetically marked vaccines to distinguish serologically between RPV- and PPRV-infected and -vaccinated animals.

The variation in immunological responses among different viruses is probably due to their different replication efficiencies in vivo. The data obtained in this study showed that the RPV-PPRFH chimeric virus is a better marker vaccine than RPV-PPRFH for PPR and appears to be as good as the conventional PPR vaccine. Also, as this virus grows to a higher titre in tissue culture than the previously produced chimeric RPV-PPRFH virus, it would be economical to produce as the conventional PPR vaccine. The deployment of a marker vaccine against PPR in the field would help greatly in control and eradication programmes. However, large-scale field trials involving a much larger number of animals of varying age, sex, breed and physiological status need to be carried out to establish further the safety of this chimeric virus before it can be used in the field.

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REFERENCES


