Introduction

Rinderpest is an extremely contagious disease of cattle, buffalo and wild ruminants with a high fatality rate in affected animals. Rinderpest has been eradicated in developed countries but it is still present in parts of Africa, such as Sudan and Somalia, and in Asia, particularly in Pakistan. Although the disease, which is caused by infection with Rinderpest virus (RPV), has for decades been one of the most widespread and important affecting cattle, its pathogenesis remains poorly understood. The virus belongs to the genus Morbillivirus in the family Paramyxoviridae and so is related to Measles virus, Canine distemper virus and Phocid distemper virus and to the morbillivi- viruses recently isolated from cetacean species (Barrett et al., 1993). The lapinized strain of the virus, RPV-L, was derived from a wild-type virus by numerous passages in rabbits (Nakamura & Miyamoto, 1953); it is avirulent in cattle and was originally developed as a vaccine to control the disease in domestic ruminants. However, it is highly virulent in rabbits causing clinical signs, including high fever, leukopenia, severe lymphoid necrosis and immunosuppression, similar to those found in cattle infected with virulent RPV (Fukuda & Yamanouchi, 1976; Imaoka et al., 1988a, b; Kobune et al., 1976a, b; Yamanouchi et al., 1974a, b). Further adaptation of the L strain to Vero cells results in a loss of virulence in rabbits (Imaoka et al., 1988a; Ishii et al., 1986) but subsequent propagation of the Vero-adapted virus in B95a cells restores this virulence (Kobune et al., 1991). Thus, the rabbit is considered to be a useful model for investigating the pathogenicity of RPV.

The most commonly used vaccine for rinderpest is the tissue culture attenuated RBOK strain, which does not cause any disease in rabbits. This vaccine was derived by repeated
passage of the wild-type parent virus (Kabete ‘O’ strain) in primary bovine kidney cells. Recently, the RPV-RBOK strain was rescued from a full-length DNA copy of its genome (Baron & Barrett, 1997). The recovery of infectious RPV from recombinant cDNAs of RPV-RBOK with exchanged components from the RPV-L strain should allow us to directly address questions concerning the molecular determinants of virulence of RPV using the rabbit experimental model. In paramyxoviruses, the surface glycoproteins, the haemagglutinin (H) and fusion (F) proteins, mediate virus attachment to, and penetration of, host cells (Scheid et al., 1972), and so these surface proteins play an important role in allowing virus entry into the host. Here we describe experiments using a virus, designated rRPV-lapH, which was rescued from a modified form of the RBOK DNA clone where the H protein gene was replaced by the corresponding gene from RPV-L. We show that the H protein plays a key role in allowing infection of rabbits by RPV, but that it does not determine pathogenicity in this species.

Methods

**Cells.** B95a cells (Kobune et al., 1990), which are highly susceptible to RPV infection (Kobune et al., 1991), were propagated in RPMI 1640 (Sigma) supplemented with 5% foetal calf serum (FCS) in a humidified atmosphere containing 5% CO\(_2\). RPMI 1640 supplemented with 2% FCS was used as maintenance medium. The antibiotics benzylpenicillin (100 U/ml) and streptomycin (100 U/ml) were used in all media.

**Viruses and rescue of recombinant virus from cDNA.** The L strain of RPV, adapted to B95a cells, was used in this study. RPV-L grown in B95a cells maintains its virulence for rabbits (Ishi et al., 1986). rRPV-RBOK was rescued from a full-length DNA copy of the genome of the RBOK vaccine strain (Baron & Barrett, 1997) which had two new restriction enzyme sites engineered into the less conserved, untranslated regions flanking the H gene to allow its replacement with the H gene from other virus strains. For this an AscI site was introduced just before the F–H intergenic region and a PmlI site just before the H-L intergenic sequence (Das et al., 2000). The H gene from the lapinized strain was amplified by RT–PCR from total RNA of RPV-L-infected B95a cells using oligonucleotide primers with the appropriate restriction enzyme sites and then inserted in place of the normal H gene into the RBOK cDNA. rRPV-lapH was rescued from this cDNA according to the procedure previously described (Baron & Barrett, 1997). The rescued viruses were passaged once in tissue culture, in B95a cells, and stored at −70 °C as the stock viruses for tissue culture infectivity studies and animal experiments.

**Rabbits, virus inocula and samples.** Three-month-old female albino rabbits (JW-NIBS strain) with an average body weight of 2.0 kg, which are highly sensitive to RPV-L (Okita et al., 1993), were obtained from the Nippon Institute for Biological Science (Ome, Tokyo). One ml each of rRPV-RBOK, rRPV-lapH and RPV-L, diluted to 10\(^{5}\) TCID\(_{50}\)/ml with maintenance medium, was intravenously inoculated into two rabbits per experiment. One control rabbit was inoculated with 1 ml of maintenance medium. The rabbits were euthanized at 3 or 6 days post-inoculation (p.i.), using Dormitor (Orion) and Dormicum (Roche), and selected tissues collected for further investigation. Other rabbits, two per virus strain, were each inoculated with 1 ml of 10\(^{5}\) TCID\(_{50}\)/ml of the three strains of RPV for analysis of the humoral antibody responses. These rabbits were euthanized at 21 days p.i. and the sera collected for antibody titration.

**Clinical investigations.** Inoculated cattle were examined daily for clinical signs of RPV infection and rectal temperatures recorded. Inoculated rabbits were also examined daily and rectal temperatures and body weights were recorded. Total white blood cell (WBC) counts in the peripheral blood of the rabbits were determined with a commercial kit (Unopette Test 58.50; Becton Dickinson).

**Virological investigations.** The rabbits were euthanized at 3 days p.i. and the lymphoid tissues (spleen, Peyer’s patch, mesenteric lymph nodes and appendix) were collected and weighed. Virus infectivity titres in 10% (w/v) homogenates of these tissues were determined in B95a cells and expressed as TCID\(_{50}\)/ml.

**RT–PCR.** Total RNA was extracted from mesenteric lymph nodes of virus-infected rabbits using a commercial reagent (Isogen; Nippon Gene). The cDNA of a part of the P gene was amplified by RT–PCR using primers PF3 and PR6 (nt 1763–1784 and 2656–2677 of the RPV-RBOK strain).

**Histopathological examination.** The various lymphoid tissues and other organs removed at autopsy were fixed in 10% formalin, dehydrated and embedded in paraffin using routine techniques. Thin sections were stained using haematoxylin and eosin.

**Virus growth.** B95a cells were infected with rRPV-RBOK, rRPV-lapH or RPV-L at an m.o.i. of 0.1 for 1 h. The virus inoculum was then removed, the cells washed once with PBS and 1.5 ml of maintenance medium was added to each well of a 12-well-plate. Samples (200 µl) for virus titration were collected immediately and at various times thereafter and stored at −80 °C. The TCID\(_{50}\)/ml of released virus was quantified by standard methods.

**Measurement of antibody titres.** The anti-RPV antibody titres in the rabbit sera were determined using an indirect ELISA. Ninety-six well microtitre plates (Nunc) were coated with 50 µl of RPV-L-infected B95a cell lysate diluted in coating buffer (0.1 M carbonate–hydrogen carbonate buffer, pH 9.6) at 4 °C overnight. The wells were blocked with 100 µl of 0.8% Block Ace (Dainihonseiyaku) in PBS at room temperature for 1 h, washed with PBS containing 0.05% Tween 20 (PBS-T) and 100 µl of diluted (from 10-fold to 10000-fold) rabbit serum was added in duplicate wells. After 2 h incubation at 4 °C, the wells were washed with PBS-T and incubated for 1 h with 100 µl of 1000-fold diluted HRP-conjugated goat anti-rabbit IgG (Tago). Following a final wash with PBS-T, 100 µl of peroxidase substrate (Bio-Rad) was added to each well and the absorbance at 655 nm measured 30 min later.

Results

**Rescue of recombinant virus and infectivity for cattle**

rRPV-RBOK and rRPV-lapH were rescued from their respective full-length cDNAs on 293 cells (Baron & Barrett, 1997). To determine whether or not the exchange of the H gene had affected the ability of the RBOK vaccine to replicate in, and protect cattle from, virulent RPV challenge, two steers were inoculated subcutaneously with rRBOK-lapH. Two weeks later they were challenged with virulent RPV (Saudi/81 strain) at a dose which normally causes death within 10 days (Ohishi et al., 2000). The two steers remained healthy throughout the experiment, following both vaccination with rRPV-lapH and challenge with the virulent virus, while a
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Fig. 1. Rectal temperatures in cattle following vaccination and challenge. Two steers (TQ85 and TQ86) age approximately 6 months were inoculated subcutaneously with $10^4$ TCID$_{50}$ of rRPV-lapH. Two weeks later they were challenged with $10^5$ TCID$_{50}$ of virulent RPV (Saudi/81 strain), a virus dose which normally causes death of the animals within 10 days (Ohishi et al., 2000). The unvaccinated challenge control animal (TQ96) was euthanized at 8 days p.i. when it began to show severe signs of rinderpest infection.

Fig. 2. Virus growth in tissue culture. B95a cells were infected with rRPV-RBOK, rRPV-lapH and RPV-L at an m.o.i. of 0.1 for 1 h. Samples of the cell medium were taken at the times indicated for determination of virus titre. Maximum titres for RPV-L, rRPV-lapH and rRPV-RBOK were observed on day 1, day 2 and day 4 p.i., respectively. For all three virus infections, the maximum titre reached was $10^4$–$10^5$ TCID$_{50}$/ml.

In vitro growth characteristics of the recombinant virus

The in vitro growth characteristics of rRPV-lapH in B95a cells were compared with those of rRPV-RBOK and RPV-L. RPV-L grew rapidly and reached its maximum titre at 24 h p.i. while rRPV-RBOK grew more slowly, reaching its maximum titre at 96 h p.i. Compared with the parental viruses, rRPV-lapH grew at an intermediate rate and took 48 h to reach its maximum titre. However, the overall growth rate of rRPV-lapH was similar to that of rRPV-RBOK and both showed a broad peak of virus production from 48 to 96 h p.i. In infections with all three viruses, the same maximum virus yield, with an infectivity titre of $10^4$–$10^5$ TCID$_{50}$/ml, was observed (Fig. 2).

Clinical signs following virus inoculation into rabbits

Rabbits inoculated with the L strain usually show pyrexia, leukopenia and failure to gain body weight. In the present study rabbits inoculated with $10^4$ TCID$_{50}$ of the L strain became pyrexic and body weight decreased from 2 days p.i. while leukopenia was observed from 1 day p.i. These clinical signs lasted until the day of euthanasia. Severe diarrhoea was also observed from 2 days p.i. in rabbits inoculated with the L strain. Rabbits inoculated with either rRPV-RBOK or rRPV-lapH showed no observable clinical signs of infection (Fig. 3). Six rabbits, two per virus strain, were each inoculated with the three strains of RPV for one experiment, and the experiment was repeated three times. As the clinical symptoms were well reproduced, representative data are shown in Fig. 3.
Table 1. Virus titres in rabbit lymphoid tissues

<table>
<thead>
<tr>
<th>Rabbit inoculated with:</th>
<th>Virus titre (TCID₅₀/ml) at 3 days p.i. in:</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Appendix</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>RPV-L</td>
<td>$10^{3.7}$</td>
<td>$10^{3.5}$</td>
</tr>
<tr>
<td>rRPV-lapH</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rRPV-RBOK</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detected.

Fig. 4. Detection of RPV RNA by RT–PCR. The RT–PCR was performed using total RNA extracted from mesenteric lymph nodes of virus-infected rabbits at 3 days p.i. The expected size of the amplified fragment was approximately 910 bp. Lanes 1, 2 and 3 indicated amplified products from each rabbit inoculated with RPV-L, rRPV-lapH and RBOK strain, respectively.

Virus growth in lymphoid tissues

The virus present in each lymphoid tissue examined post mortem was titrated. High virus titres, ranging from 3.5 to 4.3 log₁₀, were detected in the mesenteric lymph nodes, the appendix and Peyer’s patches of all rabbits inoculated with the L strain. In contrast, no virus was detected in lymphoid tissues of rabbits inoculated with either rRPV-RBOK or rRPV-lapH (Table 1). We further attempted to detect the viruses by RT–PCR. RNA was extracted from mesenteric lymph nodes of the virus-infected rabbits at 3 and 6 days p.i. Amplified products were readily detected at 3 days p.i. for rabbits inoculated with the L strain but not for those inoculated with rRPV-RBOK, a result consistent with the virus isolation data (Fig. 4). Interestingly, small amounts of RT–PCR products were amplified from tissues of rabbits inoculated with rRPV-lapH. At 6 days p.i., RT–PCR products were also detected for RPV-L-infected rabbits, but no products were detected for rRPV-RBOK- or rRPV-lapH-infected rabbits (data not shown).

Histopathology of lymphoid tissues

Tissues for histopathology were from rabbits euthanized 3 days p.i. Since the primary targets of RPV in both cattle and rabbits are the lymphoid tissues (Yamanouchi et al., 1974a; Okita et al., 1993; Duprex et al., 1999), these tissues were examined for gross pathological changes. Enlargement of mesenteric lymph nodes, superficial lymph nodes and Peyer’s patches was observed only in rabbits inoculated with RPV-L. Evidence of intestinal haemorrhage was also found in one of these animals. Histopathologically, severe necrosis, infiltration of inflammatory cells and multinuclear giant cells were observed in all the lymphoid tissues of rabbits inoculated with RPV-L (Fig. 5). In rabbits inoculated with rRPV-lapH the ‘starry sky’ effect, which indicates activation of macrophages and propagation of lymphocytes (Enriquez & Neiman, 1976), was observed. In contrast, the lymphoid tissues of rabbits inoculated with rRPV-RBOK were normal in every respect.

Antibody responses in rabbits to the different RPVs

Two rabbits were inoculated either with rRPV-RBOK, rRPV-lapH or RPV-L to determine the antibody responses, if any, to virus infection. A 100-fold lower dose of virus was used to inoculate these rabbits so that those given RPV-L would survive long enough to produce an antibody response. The rabbits were euthanized at 21 days p.i. and the sera collected to test for the presence of anti-RPV antibodies. Antibody responses to RPV were not detected in sera from rabbits inoculated with rRPV-RBOK. In contrast, rabbits inoculated with either RPV-L or rRPV-lapH produced measurable titres of anti-RPV antibodies (Table 2).

Discussion

We succeeded in rescuing a recombinant RPV in which the H gene of the RBOK vaccine was replaced with that derived from the lapinized vaccine strain (rRPV-lapH). The recombinant virus grew as well in B95a cells as the RBOK vaccine, the virus which contributed the major part of the genome, but slightly slower than the lapinized virus. The exchange of the H genes did not affect the ability of the RBOK strain to act as a vaccine to protect cattle against the most virulent challenge strain of RPV available, neither did it affect the apathogenic nature of the RBOK strain for cattle. rRPV-lapH was not
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Fig. 5. Histopathology in lymphoid tissues. Thin sections of lymphoid tissues were stained with haematoxylin and eosin. Representative lesions, in mesenteric lymph nodes, are shown for each virus (a, RPV-L; b, rRPV-lapH; c, rRPV-RBOK; × 100). (a) Severe inflammatory lesions with numerous necrotic cells and some multinucleated giant cells (white arrow); (a’) a necrotic giant cell, × 400; (b) ‘starry sky’ effect.

virulent in rabbits, as is the case for rRPV-RBOK, and the results of this study indicate that viral proteins other than the H protein are responsible for pathogenicity in rabbits. An anti-RPV antibody response was induced following infection with this virus that was not seen following infection with the rRBOK vaccine. Therefore, exchanging the H proteins did allow the RBOK virus to enter and replicate in some rabbit cells.

Histopathological examination of lymphoid tissues from rabbits infected with rRPV-lapH did not reveal the severe lesions, consisting of giant cell formation and necrosis of lymphoid follicles, observed in all rabbits infected with RPV-L. However, immunoreactive degeneration (‘starry sky’ effect) was observed in the lymphoid tissues of rabbits infected with rRPV-lapH and this result suggested that multiplication of rRPV-lapH might have taken place in these tissues, which would explain the generation of humoral antibody responses following infection with this virus. Paradoxically, rRPV-lapH could not be detected in homogenates of the different lymphoid organs from infected rabbits. It may be that growth of the virus was below detectable levels for the tests employed. The results of RT–PCR on the tissues of the infected rabbits at 3 days p.i. indicated that rRPV-lapH could grow in lymphoid tissues although the growth rate was much lower than for RPV-L. In addition, rRPV-lapH was quickly eliminated from the tissues (by 6 days p.i.), which may be due to the immune reactions implied by histopathological observation.

In the case of Measles virus (MV), the H gene of a rodent-adapted neurovirulent strain was shown to confer the ability to replicate in brain tissue on the vaccine strain of the virus in vivo. Mice infected with a recombinant vaccine whose H protein was replaced with that of a neurovirulent MV strain became clinically ill. However, the authors concluded that replacement of H gene alone is not sufficient to cause the full pathology of the neurovirulent strain since the neuropathology induced by infection with the recombinant MV was not as severe (Duprex et al., 1999). Similarly, transgenic mice expressing the CD46 receptor for MV allowed replication of the vaccine strain of
Table 2. Antibody responses to different RPV strains

<table>
<thead>
<tr>
<th>Virus strain/rabbit no.</th>
<th>Pre inoculation</th>
<th>After infection</th>
<th>Antibody response</th>
</tr>
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<tbody>
<tr>
<td>RPV-L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1/10</td>
<td>1/10000</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>&lt; 1/10</td>
<td>1/1000</td>
<td>+</td>
</tr>
<tr>
<td>rRPV-lapH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1/10</td>
<td>1/100</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>1/10</td>
<td>1/1000</td>
<td>+</td>
</tr>
<tr>
<td>rRPV-RBOK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>1/10</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>1/10</td>
<td>1/10</td>
<td>−</td>
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</tbody>
</table>

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