Structural, antigenic and immunogenic relationships between European brown hare syndrome virus and rabbit haemorrhagic disease virus

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The capsid protein of a French isolate of the European brown hare syndrome virus (EBHSV) was expressed in the baculovirus system. The recombinant EBHSV (rEBHSV) capsid protein was able to self-assemble into virus-like particles (VLPs). The VLPs were indistinguishable from the infectious EBHSV and displayed morphological characteristics similar to those we have described for the VLPs resulting from the expression of the capsid protein of rabbit haemorrhagic disease virus (RHDV), a closely related calicivirus. Cross-protection experiments showed that vaccination with rEBHSV particles did not protect rabbits against an RHDV challenge. A set of monoclonal antibodies (MAbs) was raised against rEBHSV capsid protein and used together with anti-RHDV and anti-EBHSV MAbs produced against native viruses to study the antigenic relationships between the two caliciviruses. All six anti-EBHSV MAbs delineated discontinuous epitopes; two of them reacted with specific surface epitopes and the remaining four reacted with internal epitopes which were also present in rRHDV. All anti-RHDV MAbs were monospecific; three reacted with surface linear epitope(s), two reacted with internal linear epitope(s) and one with a surface conformational epitope. On the basis of all these results, a classification of RHDV and EBHSV as two serotypes of a single serogroup is proposed.

Introduction

European brown hare syndrome virus (EBHSV), a calicivirus responsible for a highly contagious disease, was first reported in European countries in the early 1980s (Gavier-Wilden & Morner, 1991). The syndrome, a severe necrotic hepatitis, could affect up to 100% of a hare population in which animals usually died within 48–72 h (Chasey et al., 1992). EBHSV is closely related to rabbit haemorrhagic disease virus (RHDV) and the distinction between the two viruses was originally difficult to establish. They have the same morphology and affect animals within the Lagomorph family (Chasey et al., 1992). Both diseases are characterized by a high mortality rate and similar clinical signs corresponding to degeneration of hepatocytes and liver necrosis (Fuchs & Weissenböck, 1992). Antigenic relationships between the two viruses were established by using anti-RHDV monoclonal antibodies (MAbs) and polyclonal sera in a series of immunological assays (Capucci et al., 1991, 1995; Chasey et al., 1992; Wirblich et al., 1994). Because of the similarity between the two viruses, cross-species infection experiments were performed in order to test whether EBHSV and RHDV were two related but different viruses or two serotypes of the same virus. Results were controversial but most of the attempts to transmit the disease across species have failed to produce illness (Capucci et al., 1991; Chasey et al., 1992). A straightforward distinction between the two viruses was finally obtained by the determination of the RNA sequence of the EBHSV genome (Le Gall et al., 1996; Wirblich et al., 1994) and its comparison with available RHDV genome sequences (Meyers et al., 1991b; Rasschaert et al., 1995). Alignment of the sequences showed 71% nucleotide identity and amino acid alignment showed 78% identity and 87% similarity (Le Gall et al., 1996).

EBHSV and RHDV have been characterized as members of the Caliciviridae on the basis of their morphology and genome...
sequences. Caliciviruses have been isolated from many mammalian hosts including man (Seal et al., 1995) and a number of human small round-structured viruses have recently been classified in the Caliciviridae (Kapikian et al., 1996). The genome of caliciviruses is a 7.5 kb positive single-stranded RNA (Lambden & Clarke, 1995). Like the picornavirus genome, the calicivirus RNA has a VPg linked to its 5′ terminus (Burroughs & Brown, 1978; Meyers et al., 1991a). The virion is 35 nm in diameter and shows a structured surface with regularly arranged cup-shaped depressions (Prasad et al., 1994a). Within the Caliciviridae, RHDV and EBHSV are unusual in that the coding sequence of their capsid gene is part of the first ORF, which encodes a large polyprotein, whereas the capsid protein of the other caliciviruses is encoded by a subgenomic RNA (Lambden & Clarke, 1995). A subgenomic RNA of 2.2 kb, which is collinear with the 3′ terminus of the genome and covers the capsid gene, is found in both viruses (Meyers et al., 1991a; Wirblich et al., 1994). It remains unclear whether, in vivo, the mature capsid protein results from the translation of the subgenomic RNA, from the processing of the polyprotein or from both (Alonso et al., 1996; Boga et al., 1992; Sibilia et al., 1995).

Wirblich et al. (1996) recently reported a detailed characterization of the genetic map for RHDV. The authors showed that the protein VP10 encoded by ORF2 located at the 3′ end of the genome was a minor structural component of the mature virion. However, the expression of VP10 seems dispensable for the assembly of capsid particles of caliciviruses as demonstrated by the self-assembly of recombinant capsid protein to yield virus-like particles (VLPs) when expressed in the baculovirus/Sf9 insect cell system (Laurent et al., 1994; Nagesha et al., 1995; Sibilia et al., 1995; Jiang et al., 1992, 1995). In that respect, the absence of nucleic acid associated with VLPs clearly demonstrated the intrinsic property of the calicivirus capsid protein alone to self-assemble into particles. Electron cryomicroscopy analysis followed by three-dimensional resolution of calicivirus VLPs has shown that the particle is composed of 90 dimers of the capsid protein (Prasad et al., 1994b; Thouvenin et al., 1997). Production of VLPs has also been of great help for immunological and epidemiological studies of the human caliciviruses (Gray et al., 1994; Green et al., 1993; Monroe et al., 1993), but the lack of an animal model hampered the evaluation of these recombinant antigens in vaccination trials. However, in the RHDV model, VLPs resulting from the expression of the capsid protein, when tested in vaccination trials, induced a strong protective immunity in vaccinated animals (Laurent et al., 1994; Nagesha et al., 1995; Sibilia et al., 1995).

In this paper, we report the ability of recombinant EBHSV (rEBHSV) capsid protein to form VLPs by using the baculovirus/Sf9 expression system. Cross-species protection of rabbits against RHDV using rEBHSV particles as a vaccine was investigated. The rEBHSV particles were used to generate a library of anti-EBHSV MAbs, which was used together with anti-RHDV MAbs to study cross-reactivity between EBHSV and RHDV.

**Methods**

- **Viruses and cells.** Spodoptera frugiperda (Sf9) cells were maintained in suspension at 27 °C in Hink’s medium (Gibco BRL) supplemented with 10% FCS (Boehringer Mannheim). Wild-type AcRPe-SC baculovirus (as well as recombinant baculoviruses) were grown on Sf9 cell monolayers, as described by O’Reilly et al. (1992). Recombinant baculoviruses used in this study were Bac G3.12 (Laurent et al., 1994) and Bac H3.16 described below.

- **MAb libraries.** Three MAb libraries were generated, which differed in the nature of the antigen used for the immunization and screening procedures. For the first two, purified RHDV or purified EBHSV were used as antigens. For the third, mice were immunized with purified rEBHSV VLPs. The immunization regimen was identical for all three fusion experiments and has been already described (Laurent et al., 1994; Thouvenin et al., 1997). Screening for hybrids secreting antibodies was performed by ELISA. The anti-RHDV library was screened by three different ELISA tests, in order to select MAbs which could detect the virus coated on the solid phase, bind to virus captured by a polyclonal antibody or capture the virus (J.-F. Vautherot, unpublished results). The two anti-EBHSV libraries were screened by using ELISA tests in which the antigen, purified EBHSV virus or rEBHSV particle was coated on the solid phase.

All MAbs used in this study were semi-purified from ascites fluids by standard salting-out procedures in which sodium sulphate at a final concentration of 18% was used to precipitate the IgGs. After a centrifugation of 30 min at room temperature at 4500 r.p.m. (Jouan R422 centrifuge), the pellets were resuspended in PBS and extensively dialysed against the same buffer. MAbs were diluted 1:1 in glycerol and stored at −20 °C.

- **Plasmid construction and selection of recombinant baculovirus.** The procedure followed was essentially the same as previously described (Laurent et al., 1994). Briefly, inserts containing the coding sequence for EBHSV VP60, designated H3, were obtained by PCR (Perkin Elmer apparatus) with pUH 9.26 (Le Gall et al., 1996) as a template and primers 39 and 37. Oligonucleotide 39, AGATCTGGATCCATGGAGGTAAAGCCGCGCT, is located at the start codon (bold) of the capsid coding sequence and oligonucleotide 37, AGATCTGGATCCATTTATAATTCGCCCTTTAACT, is complementary to the 3′ end of the genome. A BamHI restriction site (underlined) was added in each primer. The amplification products were cloned into the transfer vector pVL941 and into pBluescript SK(−) (Stratagene) at the BamHI restriction site to produce pVLH3 and PBH3, respectively. Wild-type baculovirus AcRPe-SC DNA, previously linearized with Bsu36I, and plasmid pVLH3 were used to cotransfect Sf9 cells by using Lipofectin (Gibco BRL) (O’Reilly et al., 1992). Positive recombinant baculovirus Bac H3.16 was identified by hybridization of dot-blotted DNA and by indirect immunofluorescence staining with the anti-EBHSV MAB H42. Bac H3.16 was plaque-purified twice and used to prepare virus stocks.

- **Production and purification of recombinant virus-like particles.** To produce each of the specific VLPs, Sf9 cells were infected with recombinant baculoviruses Bac H3.16 or Bac G3.12 at a m.o.i of 10 p.f.u. per cell. For production of radiolabelled VLPs, 1 × 10^7 Bq per 10^8 cells of Pro-mix (Amersham) was added to the culture medium 3 h post-infection. The recombinant particles present in the culture medium were purified as previously described (Laurent et al., 1994). Particle integrity was examined by electron microscopy after staining with 2% uranyl
The amount of protein was estimated with a Micro BCA protein assay reagent test (Pierce).

**In vitro translation in rabbit reticulocyte lysate.** The EBHSV capsid gene cloned into plasmid pBH3 was transcribed and translated by using the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of Pro-mix according to the manufacturer’s instructions.

**Immunoprecipitation of radiolabelled proteins.** Protein samples were incubated overnight at 4°C with MAB at a final concentration of 1:500 (v/v). The samples were diluted 10 times in RIPA buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.5% Triton X-100) and protein A-Sepharose (Pharmacia) was added for an incubation period of 1 h at 37°C with gentle rotation. The beads were washed three times with RIPA buffer. Proteins were then dissociated from the beads by boiling in sample buffer (12.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS) for 3 min and further analysed by SDS–PAGE on a 10% acrylamide gel, followed by autoradiography.

**Vaccination of rabbits with recombinant VLPs.** Two groups (G1 and G2) of specific-pathogen-free, 11-week-old New Zealand White rabbits were injected intramuscularly with 300 μg purified VLPs emulsified in Freund’s complete adjuvant. The 10 rabbits in G1 received rEBHSV particles and the 5 rabbits in G2 received rRHDV particles. The 10 rabbits in the control group G3 were left untouched. All rabbits were challenged by an intramuscular injection of 1000 LD₅₀ RHDV 17 days after vaccination. Serum samples were collected on the day of vaccination, on the day of challenge (17 days after infection) and 7 days after challenge for survivors (24 days after vaccination). Antibody titres were determined by ELISA (see below) with rRHDV or rEBHSV as antigen.

**ELISA**

Indirect ELISA for antibody detection in sera of vaccinated rabbits. ELISA was done as described by Laurent et al. (1994). The A₅₆₅ of a dilution of 1:300 of the antisera was determined as a cut-off value.

Direct ELISA for antigenic cross-reactivity studies. Microtitre plates were coated with 50 ng recombinant particles diluted in PBS. The subsequent steps of blocking, washing and incubation with MABs diluted at 1:500 were performed as described previously (Laurent et al., 1994; Vautherot et al., 1992).

**Western blot analysis**

Denatured antigen. Recombinant capsid proteins produced in the baculovirus system (2–5 μg) were separated by SDS–PAGE (10% acrylamide). The proteins were electrotransferred onto nitrocellulose membranes and immunostained as described by Labbe et al. (1993), except for the following modifications. The steps of blocking, washing and incubation with MABs were performed in RIPA containing non-fat dry milk at 5%, 1% and 3%, respectively. All the MABs were used at a dilution of 1:1000.

Native recombinant particles. Purified VLPs (2 μg) were supplemented with 10% glycerol and electrophoresed on a 0.6% agarose gel in a 25 mM Tris–HCl pH 7.4–200 mM glycine buffer (TGA gel) for 5 h at 50 V. The VLPs were transferred onto nitrocellulose membranes as described by Aponte et al. (1993) and immunostained under the conditions described for denatured antigen.

**Results**

**Production and self assembly of rEBHSV capsid protein**

The positive recombinant baculovirus Bac H3.16 produced by recombination between baculovirus transfer vector and wild-type baculovirus was plaque-purified twice and tested for its ability to produce rEBHSV capsid protein. The rEBHSV protein, released into the culture medium from 36 h post-infection, was able to self-assemble into VLPs which could be purified by centrifugation on CsCl gradients and visualized by electron microscopy after negative staining (Fig. 1a). The rEBHSV particles were morphologically indistinguishable from the EBHS native virus and from recombinant RHDV (rRHDV) particles produced under the same conditions (data not shown). They were devoid of any nucleic acid as deduced from their density of 1.31 instead of 1.36 for the native virus, and from their A₂₆₀/A₂₃₀ ratio of 1.15 for the recombinant particles versus 1.44 for the virus. The yield of rEBHSV was estimated...
Table 1. Reactivity of serum samples of rabbits vaccinated with rEBHSV or rRHDV particles and then challenged with RHDV 17 days later

Data presented are $A_{405}$ values for serum dilutions of 1:300, collected 17 and 24 days after vaccination. The negative cut-off value was 0.3.

<table>
<thead>
<tr>
<th>VLPs VAC*</th>
<th>Rabbit no.</th>
<th>Day 17</th>
<th>Day 24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rRHDV†</td>
<td>rEBHSV†</td>
<td>rRHDV†</td>
</tr>
<tr>
<td>rEBHSV</td>
<td>1</td>
<td>1·11</td>
<td>0·98</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1·42</td>
<td>1·12</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt; 2</td>
<td>0·95</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1·46</td>
<td>0·56</td>
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<tr>
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<td>6</td>
<td>1·16</td>
<td>0·46</td>
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<td>7</td>
<td>&gt; 2</td>
<td>1·46</td>
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<td></td>
<td>8</td>
<td>&gt; 2</td>
<td>&gt; 2</td>
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<tr>
<td></td>
<td>9</td>
<td>1·18</td>
<td>&gt; 2</td>
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<tr>
<td></td>
<td>10</td>
<td>&gt; 2</td>
<td>&gt; 2</td>
</tr>
<tr>
<td>rRHDV</td>
<td>11</td>
<td>&gt; 2</td>
<td>0·18</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&gt; 2</td>
<td>0·15</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>&gt; 2</td>
<td>0·17</td>
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<tr>
<td></td>
<td>14</td>
<td>&gt; 2</td>
<td>0·24</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>&gt; 2</td>
<td>0·30</td>
</tr>
<tr>
<td>Controls</td>
<td>(10 rabbits)</td>
<td>&lt; 0·1</td>
<td>&lt; 0·1</td>
</tr>
</tbody>
</table>

* VLP (300 µg) used as vaccine.
† VLPs used as antigen in ELISA test for titration of collected sera.
– indicates rabbit was dead.

Antigenicity of the recombinant protein was shown to be conserved as it specifically reacted in immunoprecipitation (Fig. 1c) and in ELISA (Fig. 2a) with an anti-EBHSV hyperimmune serum (data not shown) and with MAb H42, a MAb raised against native EBHS virus.

Since a disulfide-bond dimer of rRHDV capsid protein could be resolved under non-reducing conditions (Laurent et al., 1994), radiolabelled rEBHSV capsid protein was further analysed under non-reducing conditions after immunoprecipitation with MAb H42 (Fig. 1c). In the absence of a reducing agent, a high-molecular-mass protein of 130 kDa, which could correspond to the dimeric form of the capsid protein of rEBHSV, was immunoprecipitated in addition to the monomeric form. The same high-molecular-mass product was observed when the EBHSV capsid protein was produced by in vitro translation in rabbit reticulocyte lysate and further analysed under the same conditions (Fig. 1c). The smaller additional products observed after in vitro translation of the EBHSV capsid gene were probably due to a classical internal initiation at an upstream AUG codon.

Cross-protection assay

The high degree of homology between the two capsid proteins, with 76% identical amino acids, together with the
Table 2. Summary of Western blot reactivity of MAb libraries against denatured and non-denatured rRHDV and rEBHSV particles

Reactivity was scored visually on a scale of + to +++. ++/− indicates an ambiguous result.

<table>
<thead>
<tr>
<th>MAb*</th>
<th>rRHDV</th>
<th>rEBHSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Denatured†</td>
<td>Native†</td>
</tr>
<tr>
<td></td>
<td>Denatured†</td>
<td>Native†</td>
</tr>
<tr>
<td>Anti-RHDV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI (surface linear)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>+ +</td>
<td>+ + +</td>
</tr>
<tr>
<td>D23</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>C36</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>GII (surface conformational)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E29</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>GIII (internal linear)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A47</td>
<td>+ + + +</td>
<td>−</td>
</tr>
<tr>
<td>B45</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Anti-EBHSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GII (surface conformational)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H42</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Anti-rEBHSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GII (surface conformational)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W42</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>GIV (internal conformational cross-reactive)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y45</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Q6</td>
<td>+ /−</td>
<td>−</td>
</tr>
<tr>
<td>Z33</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>O4</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Groups of MAbs classified according to the nature of the epitopes recognized.
† Western blot on recombinant particles separated by SDS–PAGE under denaturating conditions (boiled, β-mercaptoethanol, SDS).
‡ Western blot on recombinant particles separated on TGA gels under non-denaturating conditions.

existence of common epitopes on both viruses, prompted us to investigate the ability of rEBHSV particles to protect rabbits against RHDV. The vaccination procedure was essentially similar to the vaccination test previously described with rRHDV particles (Laurent et al., 1994). Rabbits vaccinated with rRHDV survived until they were sacrificed on day 24, and no clinical signs of the disease could be observed in these animals. Serum specimens of these rabbits exhibited a high level of anti-rRHDV antibodies (all sera with $A_{4/05} \geq 1$ and 5/10 with $A_{4/05} > 1$) on day 17. The 10 control rabbits died of the disease within 48 h following exposure to the virus. None of these rabbits seroconverted either against RHDV or against EBHSV (10 sera with $A_{4/05} < 0.1$).

**Antigenic cross-reactivity between rEBHSV and rRHDV particles**

The antigenic relationships between the viruses were further characterized by using MAbs selected from three different libraries. From the first anti-RHDV MAb library, 38 stable hybridomas were cloned from 60 hybrids that scored positive. The MAbs E3 (IgG2a), D23 (IgM), E29 (IgG2a), A47...
group reacted with denatured as well as native particles into three groups. The MAbs E3, C36 and D23 in the first determined by Western blotting allowed us to classify them of six (E3, E29, C36 and D23) were highly reactive (all IgG2a-type) were found to be moderately reactive (0 ± 7 < A450 < 1 ± 2) (Fig. 2a). These MAbs were found to be cross-reactive with rRHDV in ELISA (Fig. 2a). It was of interest to determine the MAbs that showed cross-reactivity to RHDV and EBHSV. When tested for their reactivity against both recombinant particles, anti-RHDV MAbs were found to be monospecific whatever epitope they recognized. However, all anti-EBHSV MAbs showing reactivity with an internal conformational epitope (Y45, Q6, Z33 and O4) were found to be cross-reactive with rRHDV in ELISA (Fig. 2a).

Discussion

The baculovirus expression system has been proven to allow high level production of functional recombinant RHDV capsid proteins (Laurent et al., 1994; Nagesha et al., 1995; Sibilia et al., 1995). Similar results could be expected, and indeed were found, when the capsid protein of the closely related EBHS calicivirus was produced in the same system. The recombinant capsid protein was produced in large amounts and released from the infected Sf9 cells into the supernatant from which assembled VLPs were purified. These VLPs were morphologically indistinguishable either from the parent virus or from rRHDV particles. As described for rRHDV particles, many EBHSV particles appeared to be electron-dense. However, the A290/A280 ratio and the density of the recombinant particles demonstrated that they were devoid of any nucleic acid. A recent analysis of cryoelectron microscopy images of the rRHDV particles clearly showed that they contained no nucleic acid (Thouvenin et al., 1997). The electron-dense core could be due to a very tight structure resulting from the association of the capsid protein to build the particles. The molecular mass of the EBHSV capsid protein appeared to be greater than predicted, 65 kDa instead of 60 ± 4 kDa, and greater than the molecular mass of the RHDV capsid protein (estimated at 62 kDa). The same pattern was observed when the two protein genes were translated in vitro (data not shown) and was also reported when the EBHSV and RHDV capsid genes were expressed in Escherichia coli (Wirblich et al., 1994), thereby excluding a eukaryotic post-translational modification.

The assembly process of caliciviruses is still under investigation. Structural studies based on cryomicroscopy image analysis showed that caliciviruses are composed of 90 dimers of the capsid protein (Prasad et al., 1994a), but the role of dimerization in the initial step of virus assembly is not known precisely, nor is the mechanism(s) by which the monomers interact. The immunoprecipitation of purified radiolabelled EBHSV particles by the anti-EBHSV MAb H42, followed by analysis under non-reducing conditions, revealed a product of 130 kDa. We have previously reported similar results for rRHDV capsid protein (Laurent et al., 1994). These data

<table>
<thead>
<tr>
<th>Anti-RHDV</th>
<th>Anti-EBHSV</th>
<th>Anti-rEBHSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3</td>
<td>W42</td>
<td>H42</td>
</tr>
<tr>
<td>D23</td>
<td>Y45</td>
<td>W42</td>
</tr>
<tr>
<td>E29</td>
<td>Q6</td>
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<td>Z33</td>
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<tr>
<td>B45</td>
<td></td>
<td>O4</td>
</tr>
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</table>

Table 2, Fig. 3. The MAb E29 in the second group was only reactive with native particles suggesting that it recognized a surface conformational epitope. The MAbs A47 and B45 in the third group were only reactive with denatured particles suggesting that they recognized an internal linear epitope. (Table 2, Fig. 3).
suggest that the formation of disulfide-linked dimers is involved in the oligomerization process of the EBHSV and RHDV capsid proteins. As both the EBHSV and the RHDV capsid protein have only one cysteine residue, the position of which is not conserved between the two proteins (Cys-274 for RHDV and Cys-135 for EBHSV), the formation of a dimer sensitive to reducing agents could proceed through only one possible intermolecular disulfide bond. It should be stressed that disulfide dimers coexist with monomers, a result reported for other disulfide-linked proteins (Sapp et al., 1995). The presence of monomers could be explained by the co-purification of a significant quantity of monomers with the VLPs (Thouvenin et al., 1997); however one cannot exclude their participation in the formation of a trimer. The production of a recombinant capsid protein without cysteine to further study the oligomerization of the calicivirus capsid protein is in progress.

The production of a properly folded rEBHSV protein greatly facilitated the straightforward demonstration of the absence of cross-protection between EBHS and RHD caliciviruses. Even when injected with a larger amount of rEBHSV, 300 µg of rEBHSV versus 100 µg of rRHDV (Laurent et al., 1994), rabbits vaccinated with rEBHSV particles were not protected against a virulent RHDV challenge. Previous cross-protection experiments, performed with native viruses, yielded quite different results, as a partial protection of EBHSV-vaccinated rabbits against a RHDV challenge was reported (Chasey et al., 1992). It should be stressed that in our experimental protocol, rabbits were challenged with 1000 LD<sub>50</sub> after a single immunization. Such a challenge might correspond to more demanding experimental conditions than those described by Chasey et al. (1992). It would have been of interest to test the ability of both recombinant particles to elicit a protective response against EBHSV infection in hares. However, the difficulties encountered in the reproduction of the disease in hares have, until now, hampered the realization of this reciprocal test.

As the humoral response was suspected to play a key role in protection against RHDV (Parra & Prieto, 1990; Laurent et al., 1994), we monitored the seroconversion of the vaccinated animals. All rabbits vaccinated either with rRHDV or rEBHSV particles developed high anti-RHDV antibody titres. However, it seems likely that antibodies to RHDV elicited by vaccination with rEBHSV particles were devoid of neutralizing activity, in contrast to those induced by the injection of rRHDV particles. Data on the precise localization of neutralizing epitopes on RHD or EBHS viruses are not yet available, although it seems likely that virus-specific neutralizing epitopes are exposed on the surface of the particle, whereas cross-reactive non-neutralizing epitopes are buried in the more conserved internal shell domain of the particle (Capucci et al., 1995; Chasey et al., 1992). Capucci et al. (1991) reported that the degree of proteolytic degradation was notably higher in purified preparations of EBHSV than RHDV. Since we also observed more degraded particles in rEBHSV preparations than in rRHDV preparations, we hypothesize that the anti-RHDV antibodies found in large quantities in the sera of rabbits vaccinated with rEBHSV could be directed against buried cross-reactive epitopes that became accessible on the degraded rEBHSV particles. The absence of anti-EBHSV antibodies in animals vaccinated with rRHDV is consistent with this hypothesis, assuming that by day 17 antibodies were raised only to the neutralizing epitopes exposed on the outside of the intact rRHDV particles. The presence of cross-reacting antibodies by day 24 could be explained either by a strong and rapid immune response to the degraded antigens present in the semi-purified virus stocks used for the challenge, or by the initiation of a response to internal cross-reactive antigens which became accessible upon limited replication of the virus. However, one cannot exclude a difference in the kinetics of appearance of antibodies, which would result in the appearance of the neutralizing specific antibodies before the non-neutralizing cross-reactive ones.

Production of MAbs directed against EBHSV, native virus and recombinant particles was undertaken to further study the antigenic relationships between RHDV and EBHSV. The anti-EBHSV and anti-RHDV MAbs were used in three different tests, an ELISA and two Western blot assays, either against native or denatured recombinant particles. The MAbs were classified into different groups according to the epitope recognized. All anti-rEBHSV MAbs, as well as the anti-EBHSV MAb H42, recognized conformational epitopes. The MAbs H42 and W42 reacted with surface conformational epitopes while the other MAbs recognized internal conformational epitopes and were found to be cross-reactive to RHDV particles. These findings were in agreement with the hypothesis which proposed the localization of cross-reactive epitopes in the internal shell domain of the particle (Capucci et al., 1995; Chasey et al., 1992). The anti-RHDV MAbs defined three different types of epitopes: surface linear epitopes (E3, D23, C36), surface conformational epitopes (E29) and internal linear epitopes (A47, B45). Thus, with two exceptions, the epitopes of anti-RHDV MAbs were localized on the surface of the particle. It should be noted that in our test the anti-RHDV MAb E3, displaying neutralization activity in an in vivo test, was found to recognize a surface linear epitope. This result is in agreement with cryoelectron microscopy analysis of the MAb E3 binding on the VLPs which showed a binding of only one Fab per dimer arch exposed on the surface (Thouvenin et al., 1997).

Taken together, the data obtained from cross-protection experiments and antigenic analysis confirm that most of the neutralizing epitopes are located on the surface of the particle and are specific to each virus, whilst non-neutralizing cross-reactive epitopes are buried inside the virion.

Comparison of the amino acid sequences of the EBHSV and RHDV capsid proteins showed an overall homology of 76% (Wirblich et al., 1994). While the N-terminal half of the proteins
(residues 1–297) exhibited a high degree of identity (84%), the C-terminal half of the protein (residues 298–579) was found to be more divergent (56% identity). It should be noted that the homology between the RHDV and EBHSV capsid proteins (76%) is higher than the homology between serotypes 1 and 4 of San Miguel sea lion virus (73%) (Neill, 1992). Amino acid alignment of other calicivirus capsid proteins together with the structural resolution of the virion suggest that the N-terminal half of the protein forms the internal core of the virion where cross-reactive epitopes might be localized, while the C-terminal half of the protein forms the protruding arches of the virion (Prasad et al., 1994b). The latter part of the protein would be exposed and could be relevant to antigenic variability. Consistent with this prediction is the mapping of two neutralizing epitopes of feline calicivirus in the C-terminal half of the capsid protein (Milton et al., 1992). A highly divergent domain (33% identity) of 27 amino acids (residues 407–434) that might be responsible for antigenic variability was found within the C-terminal region of the EBHSV and RHDV proteins. It should be noted that the first 19 amino acids of this region are located at the C-terminal end of region E, whilst the last 8 amino acids overlapped region F, two regions defined by Neill (1992) by amino acid alignment of animal calicivirus capsid proteins. Region E was proposed to be responsible for antigenic variability. It would be of interest to localize epitopes recognized by the different MAbs used in these studies, and especially the neutralizing anti-RHDV MAb E3, to confirm this hypothesis. For that purpose, different parts of the capsid protein could be deleted and the modified protein expressed in the baculovirus system. Furthermore, a protein chimera of RHDV and EBHSV capsid proteins expressed in the same system could be of great help.

Taking into account the inability of rEBHSV particles used as a vaccine to protect rabbits against RHDV, the antigenic relationships between RHDV and EBHSV, and the high homology in amino acid sequence between the capsid proteins of these viruses, we propose the classification of RHDV and EBHSV as two representative serotypes of a single serogroup within the Caliciviridae.

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References


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