Purification and Characterization of a Calicivirus as the Causative Agent of a Lethal Hemorrhagic Disease in Rabbits

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The causative viral agent of a lethal rabbit hemorrhagic disease has been purified and characterized. In negative-stained preparations, the virions were icosahedral, measured 27 to 35 nm in diameter, were without an envelope, and showed 10 peripheral cup-shaped depressions. The major structural protein was 60 kilodaltons, which constitutes a unique characteristic of the Caliciviridae.

In the spring and winter of 1984, outbreaks of an apparently new viral disease in rabbits were observed in the People’s Republic of China (province of Jiangsu). The characteristic pathological signs were hemorrhages in the respiratory system, liver, spleen, cardiac muscle, and occasionally in the kidneys. The disease incubation period in rabbits is 2 to 3 days, morbidity approaches 100%, and mortality is over 90% in adults. Outbreaks of a disease showing clinical signs similar to those described by Liu et al. (9) have been reported in several countries, and the disease is known as rabbit viral sudden death in Korea (7), haemorrhagic pneumonia (15) or haemorrhagic viral disease in China, and X disease of the rabbit in Italy (4). The disease has also recently appeared in the Federal Republic of Germany (10), France (11), and Spain (1, 13). Consequently, the origin of the rabbit hemorrhagic disease virus (RHDV; tentative name) is a matter of speculation, though it was first discovered in China in Angora rabbits imported from the Federal Republic of Germany (W. Xu, N. Du, and S. Liu, 4th World Rabbit Congress, 456–461, 1988).

The nature of the causative agent and therefore the correct classification of RHDV are still being disputed. The agent has been described by different authors among the picornaviruses (15), parvoviruses (Xu et al., 4th World Rabbit Congress), or caliciviruses (14).

In an attempt to positively identify the causative agent, we have purified RHDV from rabbit liver specimens collected during an outbreak of hemorrhagic disease in Asturias (northern Spain) and have characterized the virus with specific emphasis on the viral proteins, which in this case can be a relevant characteristic for classification. The viral polypeptides that reacted with convalescent rabbit antisera have also been investigated by using Western immunoblot analysis.

Liver specimens for the isolation of RHDV were obtained from animals from a farm on which 80% of the adult animals died after showing clinical signs of hemorrhagic disease (1, 9). The initial liver homogenate used had a high hemagglutination titer on group O human erythrocytes, as described for the isolates made in China (12).

The cell extracts were layered onto 20 ml of 30% (wt/vol) sucrose in 10 mM Tris hydrochloride (pH 7.5) and centrifuged in a Beckman SW28 rotor (27,000 rpm, 2 h, 4ºC). The virus pellet was resuspended in 10 mM Tris hydrochloride (pH 7.5). CsCl was added (0.46 g/ml of final sample volume), and the mixture centrifuged to isopycnic equilibrium. The viral particles banded at a density of 1.365 g/ml in the range estimated for the agent of vesicular exanthema of swine (12) or caliciviruses isolated from pinnipeds (17). Freshly prepared virus had a maximum UV absorption at 260 nm, and the A260/A280 ratio, an indicator of RNA content, was 1.44.

The virus could not be propagated in primary cultures nor in established cell lines.

The resulting purified RHDV virions were spotted on a coated electron micrograph grid and stained for 30 s with 2% potassium phosphotungstate (pH 7.2), blotted dry, and examined with a Zeiss electron microscope at 80 kV. The results from this study suggested that RHDV was structurally related to the calicivirus family (18). The virions were approximately round, icosahedral, 27 to 35 nm in diameter, without an envelope, and they showed dark areas on their surfaces (Fig. 1).

The structural polypeptides of RHDV were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12% slab gels in a discontinuous buffer system (6), and their approximate molecular sizes were estimated by direct comparison with reference markers after Coomassie blue staining. A major and several minor distinct polypeptide bands were visualized; molecular masses were approximately 60 kilodaltons (kDa) for the major component (upper arrow in Fig. 2) and 85, 56, 40, and 30 kDa for the minor components. A minor component of less than 20 kDa (lower arrow in Fig. 2) was also visible. These data showed that the structural protein of RHDV was mainly represented by a single major polypeptide species of 60 kDa, which constitutes a distinctive property of caliciviruses that is not shared by other small viruses of vertebrate origin (3).

The viral polypeptides were separated by SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and probed with convalescent or normal rabbit sera (Fig. 3). Only the polypeptides with molecular masses of 60 and <20 kDa, labeled with arrows in Fig. 2, were detected when probed with a convalescent-phase rabbit serum (Fig. 3, lane 1); there were no polypeptides detected using normal serum from uninfected animals (Fig. 3, lane 2). The strongest reaction was against the structural polypeptide VP60, supporting its role as the major capsid component. Western analysis also identified a minor polypeptide (lower arrow in Fig. 2), which may represent a degradation product of the VP60 main antigen or it may be a minor component of the virion, whose presence has been proposed for other calici-

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viruses (2, 3). Polypeptides with molecular masses of 85, 56, 40 and 30 kDa (Fig. 2) were not detected by the convalescent serum and may represent contaminants of the preparation or minor nonantigenic viral components. Similar results were obtained (data not shown) when the blots were probed with several sera from contact animals, which survived at a farm on which the rest of the rabbits died of hemorrhagic disease. None of the normal rabbit sera, obtained before the outbreak was detected, gave positive identification of VP60.

Due to the fact that the serum samples used in Fig. 3 were obtained from different animals and therefore could give rise to misleading conclusions, attempts have been made to demonstrate whether survivors of experimental infections seroconverted to the two viral proteins. Only one animal survived of 15 infected rabbits, showing that under experimental conditions mortality could approach 100%. The preimmune and immune sera were then used in Western blot experiments which yielded the results shown in Fig. 3.

Interestingly, the survivor was resistant to a subsequent nasal challenge with a dose of purified RHDV which was able to kill an unexposed rabbit. The antibody titers against VP60 of the survivor, measured in an indirect enzyme-linked immunosorbent assay, increased by 2 orders of magnitude after the challenge. Protection against RHDV has also been achieved by parenteral immunization of rabbits with purified VP60.

The results obtained suggest that seroconversion to VP60 correlated with acquired immunity against RHDV and that this viral polypeptide appears to be a good candidate for a subunit vaccine against rabbit hemorrhagic disease.

In order to characterize further the major RHDV capsid component, VP60, it was electrotransferred onto polyvinylidene difluoride filters (Immobilon; Millipore Corp.) and subjected to microsequencing (8), using an Applied Biosystems sequenator model 477A equipped with an on-line phenylthiohydantoin analyzer model 120A (5). No phenylthiohydantoin derivatives were obtained after several attempts, suggesting that the NH₂ terminus might be blocked, as has been described also for the major polypeptide of caliciviruses (16).

The viral genome was extracted from CsCl-purified RHDV as follows. SDS was added to 1%, and the mixture was heated to 50°C for 5 min. The resulting solution was diluted five times with diethylypyrocarbonate-water and treated with 1 mg of protease K per ml in 0.015 M sodium citrate-0.15 M sodium chloride (1 x SSC) at 37°C for 30 min. The nucleic acid was finally obtained by extraction with phenol-chloroform and was precipitated by adding cold absolute ethanol. The nucleic acid pellet was destroyed by treatment with 5 µg of DNase-free RNase A per ml in 2 x SSC for 15 min at 25°C, showing that the viral genome was a single-stranded RNA molecule. The undigested material gave a unique band with lower mobility than a 7.4-kilobase RNA marker (Boehringer Mannheim Biochemicals) when analyzed in a denaturing formaldehyde-agarose gel (data not shown). The genomic RNA was polyadenylated, as deduced from the synthesis of cDNA by priming with oligo(dT). The cDNA produced is currently being used for mapping and
sequencing the gene coding for the major structural polypeptide, VP60 (F. Parra and M. Prieto, unpublished data).

The evidence presented in this report, especially discovery of a single major polypeptide of 60 kDa, supports the classification of the etiological agent of rabbit hemorrhagic disease among the Caliciviridae. Therefore, according to the nomenclature rules, we propose the use of the term rabbit calicivirus for this new animal virus.

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LITERATURE CITED