Monoclonal antibodies to rabbit haemorrhagic disease virus and their use in the diagnosis of infection

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Hybridomas producing monoclonal antibodies (MAbs) to rabbit haemorrhagic disease virus (RHDV) were prepared. Using Western blot (WB) analysis, the MAbs obtained were divided into two groups, one reacting with the major structural proteins of $M$, 61K and 38K, and the other giving negative reactions. Both groups of MAbs, however, reacted specifically with RHDV in ELISA and by immunoperoxidase (IP) and immunofluorescence (IF) tests with infected cells. As demonstrated by WB using RHDV-specific MAbs and a MAb to feline calicivirus (FCV) strain F9, the major structural (capsid) proteins of RHDV and FCV have very similar sizes ($M$, 61K and 38K compared to 62K to 64K and 40K respectively). No cross-reactions of MAbs with proteins of the other virus were observed in WB analysis, ELISA, IP tests or IF. The high specificity and sensitivity of RHDV-specific MAbs make them suitable for the routine IP and IF diagnosis of RHDV in liver cells of rabbits dying after natural or experimental infections.

Introduction

Rabbit haemorrhagic disease (RHD), which emerged in China in 1984 and in European countries in 1987 to 1988, has a great economic impact, causing 60 to 100% mortality in rabbit colonies. The causal agent was demonstrated to be a virus (RHDV) with a diameter of 28 to 38 nm (Liu et al., 1984; Deng et al., 1987; Wei et al., 1987; Ohlinger et al., 1989; Šmíd et al., 1989). Several reports (Liu et al., 1984; Gu et al., 1986; Šmíd et al., 1990) have described the production of inactivated vaccines with reliable protective effects, and haemagglutination and haemagglutination inhibition tests (HT and HIT respectively) were developed for the demonstration of the virus and specific antibodies (Liu et al., 1984; Pu et al., 1985; Du et al., 1986; Ohlinger et al., 1989). The earlier, rather controversial reports concerning the classification of RHDV (Liu et al., 1984; Du et al., 1986; Shen et al., 1986; Xu et al., 1988) were followed by several papers suggesting, on the basis of morphology, the size of the major structural proteins and density in CsCl, that RHDV is a calicivirus (Du et al., 1986; Deng et al., 1987; Ohlinger et al., 1989; Šmíd et al., 1989; Valíček et al., 1990). On the other hand, RHDV differs from known caliciviruses in several respects: its haemagglutinating activity and failure in all attempts to propagate it in vitro (Studdert, 1978; Cubitt, 1987; Carter et al., 1989).

Although the results obtained hitherto suggest a fair specificity of the HIT and HT, non-specific reactions have been also observed occasionally. HIT is less sensitive and a drawback of both tests is their dependence on a continuous supply of fresh erythrocytes. An ELISA for the determination of antibodies to RHDV in rabbit blood was described recently (Rodák et al., 1990), which eliminates the drawbacks of HIT and allows a standard, sensitive and specific assessment of the immune response to infection or vaccination.

Hybridomas producing monoclonal antibodies (MAbs) to RHDV proteins were prepared with the aim of replacing HT with a more specific method. Methods for checking the specificity of MAbs as well as their possible use for the diagnosis of RHD are discussed in this paper.

Methods

*Animals.* Inbred BALB/c mice were employed for the preparation of hybridomas and ascitic fluids, as well as sources of RHDV-negative and -positive sera. Negative rabbit sera were collected in non-infected colonies, free of naturally acquired antibodies. Organs of normal rabbits and rabbits dying after infection were used for the preparation of RHDV-negative and -positive tissue sections, impressions and smears for further examination. A reference serum obtained from China, and rabbit hyperimmune and convalescent sera were used as RHDV-positive controls.

*Virus purification.* RHDV was purified from organ homogenates of rabbits dying after experimental infection with the strain CAPM V-351 [Collection of Animal Pathogenic Microorganisms (CAPM), Brno, Czechoslovakia]. The homogenates were extracted with chloroform and ultracentrifuged onto a CsCl cushion, the final step being affinity chromatography (Rodák et al., 1990). The vaccine strain F9 (CAPM) of feline calicivirus (FCV) was propagated in the Crandell feline...
kidney cell line (CRFK) in MEM. After removal of cell debris from the culture medium, the virus was concentrated by ultracentrifugation (Beckman SW28, 90 min at 27000 r.p.m.) and the pellet was resuspended in 1/100 of the original volume of phosphate-buffered saline (PBS). The suspensions of RHDV or FCV were diluted 1:500 or 1:100, respectively, with 0.05 M-carbonate–bicarbonate buffer pH 9.6 for ELISA.

**Blood sera and conjugates.** Specific antibodies were separated from swine hyperimmune sera to rabbit and mouse IgG (SwAR1gG, SwAMolGlG) by affinity chromatography (CNBr-activated Sepharose 4B, Pharmacia). The IgG fraction was separated from mouse ascitic fluid containing MAbs to RHDV (E7/C3). The antibodies were conjugated with horseradish peroxidase (HRP, RZ = 3-0, Boehringer Mannheim) by the periodate method (Boorsma & Streefkerk, 1979). Stock conjugate solutions (HRP–SwAR1gG, HRP–SwAMolGlG, HRP–RHDV MAb), containing 2 mg/ml IgG, were diluted 1:1000 to 1:2000 with PBS containing 0.1% Tween 80 and 1% lactalbumin hydrolysate (PBST–LAH) before use in ELISA or Western blot (WB) analysis. The IgG fraction of SwAMolGlG separated by ion-exchange chromatography was conjugated with fluorescein isothiocyanate (FITC–SwAMolGlG) as described by The & Feltkamp (1970).

**ELISA.** Microtitre plates (Koh-I-Noor) with wells coated with purified RHDV or FCV were incubated with the examined sample (rabbit or mouse serum, ascitic fluids, hybridoma culture medium) at 37 °C for 1 h. A sample of the ascitic fluid of FCV MAb 1 G9 was kindly supplied by Dr M. J. Carter of the University of Newcastle-upon-Tyne, U.K. One volume of sample was diluted with one (culture media), or 100 (sera, ascitic fluids) volumes of PBST–LAH and twofold dilution series were prepared for antibody titrations. A second incubation followed with either HRP–SwAR1gG (rabbit samples), or HRP–SwAMolGlG (mouse samples) under the same conditions. The results were read spectrophotometrically at 492 nm (Tietertek MCC, Lab-system) 1 h after the addition of substrate solution (5-aminosalicylic acid). Wells containing negative rabbit or mouse sera served as controls and rows of wells containing PBST–LAH alone as blanks (Rodák et al., 1990). The highest dilution giving an absorbance higher than 0.1 was considered as the final antibody titre.

**Hybridomas and tests on MAbs.** Inbred BALB/c mice were repeatedly inoculated subcutaneously with an emulsion of purified RHDV in Al–Span-Oil adjuvant (Sevac). Three to 4 days prior to fusion, the mice were inoculated intraperitoneally with a suspension of RHDV in PBS. Spleen cells were fused with myeloma cells (SP 2/0, Institute of Molecular Genetics, Prague, Czechoslovakia) at a ratio of 5:1 in 50% polyethylene glycol 1500 in serum-free Dulbecco’s MEM (DMEM, Serva) (Galfrié & Milstein, 1981). Culture media from the hybridomas grown in DMEM–HAT were examined for the presence of antibodies by ELISA 10 to 12 days later. Antibody-producing hybridomas were cloned and transferred into the complete medium RPMI 1640 and antibody production was tested again by ELISA and Western blotting. Selected hybridomas were injected intraperitoneally into Pristane (Sigma)-primed BALB/c mice (1×10⁶ cells per animal). Ascitic fluids were collected 10 days later and stored at 4 °C with 0.1% sodium azide as preservative. Specific immune rabbit sera to individual types of heavy and light chains of mouse immunoglobulins (ICN Immunobiocemicals) were used for the exact classification of the MAbs.

The concentration of immunoglobulins in ascitic fluids was determined by radial immunodiffusion in 1.5% agarose gels. Immunoglobulins, which had been separated from the same ascitic fluids and whose IgG concentration had been determined spectrophotometrically (E₂₈₀ = 14), were used as standards.

**PAGE and WB analysis.** The major structural proteins of RHDV and FCV were determined by discontinuous SDS–PAGE under reducing conditions in a 10% gel as described by Laemmli (1970). Low Mₙ standards (Pharmacia) were used for the determination of Mₙ. After electrophoresis, the gel was divided into two parts. One was stained with silver (Heukeshoven & Dernick, 1985) and proteins from the other one were transferred onto a nitrocellulose membrane (NC) (Sartorius SM 113) and used for WB analysis. Strips of NC were incubated with rabbit or mouse sera or ascitic fluids, diluted 1:1000 with PBST–LAH, at 20 °C for 1 h and subsequently with HRP–SwAR1gG or HRP–SwAMolGlG under the same conditions. After a short period of staining in substrate solution [3,3-diaminobenzidine (DAB), Merck], the reaction was stopped with 0.5% sodium azide and the strips were dried and photographed.

**Demonstration of virus by immunoperoxidase (IP) and immunofluorescence (IF) tests.** Cryostat sections, impressions and smears of liver, kidney, spleen and lung of several tens of rabbits dying after natural or experimental infections were examined. The dried samples were fixed with acetone at 20 °C for 15 min. For the IP test, the samples were pre-incubated for 15 to 30 min in PBS containing 0.1% sodium azide and 0.3% hydrogen peroxide to inhibit the activity of endogenous peroxidase (Li et al., 1987). This step was followed by incubation in a wet chamber with MAbs (ascitic fluids) in a series of 10-fold dilutions (10⁶ to 10⁰) with PBST–LAH at 20 °C for 30 min. Parallel samples incubated with serial dilutions of negative mouse serum served as controls.

After rinsing with PBS (three times for 5 min), the samples were incubated under the same conditions with HRP–SwAMolGlG or FITC–SwAMolGlG diluted 1:10 to 1:200. The samples for the indirect IP test were examined under a conventional microscope after a short incubation with the substrate solution (DAB) and a fluorescence microscope (Fluoval) was used for the indirect IF test. Other samples were incubated (30 min at 20 °C) with HRP–RHDV MAb diluted 1:20, 1:50 and 1:100 with PBST–LAH for direct IP demonstration of RHDV. Samples of organs of a healthy, non-infected rabbit were used as controls.

The same procedure was used for the indirect IP demonstration of FCV in monolayers of the cell line CRFK 6 h after infection with the strain F9. Parallel samples incubated with normal mouse serum and samples of non-infected cells served as controls.

**Results**

**Antibody titration by ELISA.** The reference serum to RHDV obtained from China, and convalescent and hyperimmune rabbit sera with ELISA antibody titres of 25600 to 204800 (Table 1) served as standards in ELISA and WB analysis. The highest dilutions giving positive absorbance values (>0-1) were 12800 and 102400 to 819200 for the mouse serum and ascitic fluid (RHDV MAbs) samples, respectively. No antibodies to RHDV were demonstrable in the normal mouse serum and the ascitic fluid of FCV MAb 1G9 by ELISA in any dilution (Table 1).

High titres of antibodies to FCV were found in the ascitic fluid containing FCV MAb 1G9 (1638400), whereas negative results were obtained with all RHDV MAb-containing samples (Table 1). Of the rabbit sera examined, only those collected from vaccinated animals showed positive titres of antibodies to FCV. However,
none of the ELISA-positive rabbit samples reacted in WB or IP with FCV (Table 1, Fig. 3).

PAGE and Western blotting

Proteins of RHDV and FCV were analysed after fractionation by PAGE. One part of the gel was stained with silver and the other was used for WB analysis.

Strips of NC membranes containing both RHDV and FCV proteins were incubated with RHDV MAb F2 and/or FCV MAb 1G9. The results of comparative examinations both confirmed the specificity of MAbs and demonstrated the similarity in size of the RHDV and FCV major proteins (RHDV, \( M_r \) values of 61K and 38K; FCV, \( M_r \) values of 62K to 64K and 40K) (Fig. 1).

Only three of the five RHDV MAbs reacted with the major RHDV proteins of \( M_r \) 61K and 38K. The specificity of the reactions was confirmed by parallel WB analysis of the rabbit sera. Neither FCV MAb 1G9 nor any of the negative control sera reacted with RHDV (Table 1, Fig. 1 and 2) and none of the RHDV-positive rabbit sera or RHDV MAbs reacted with FCV proteins. FCV MAb 1G9 reacted strongly with the FCV protein of \( M_r \) 62K to 64K, whereas the reaction with the 40K protein was markedly weaker (Table 1, Fig. 1 and 3).

Demonstration of RHDV and FCV by IF and IP tests

Examinations of organs of rabbits dying after natural or experimental RHD infections have shown repeatedly that liver contained more virus than other organs and that smears gave more reliable results than impressions or sections. Therefore liver smears were used preferentially for routine examinations.

RHDV could be observed by indirect IP and IF up to the highest dilution of MAbs used \((10^{-5})\), but the number of infected cells and the intensity of staining were indirectly proportional to the dilution. Ascitic fluids and

Table 1. Specificity of rabbit sera, mouse sera and MAbs in reactions with RHDV and FCV, tested by ELISA, WB and IP demonstration of viruses in infected cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immunization (IgG concentration)</th>
<th>Sample no.</th>
<th>Directed to</th>
<th>Reaction with RHDV</th>
<th>Reaction with FCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit blood sera</td>
<td>3 x v.</td>
<td>7/88-4</td>
<td>RHDV</td>
<td>ELISA titre</td>
<td>ELISA titre</td>
</tr>
<tr>
<td>Unknown</td>
<td>China</td>
<td>2786</td>
<td>RHDV</td>
<td>204800 ++ + + +</td>
<td>3200 ++ + + +</td>
</tr>
<tr>
<td>i. + i.</td>
<td>v. + chall.</td>
<td>2823</td>
<td>RHDV</td>
<td>51200 ++ + + + + +</td>
<td>ND Neg.</td>
</tr>
<tr>
<td>Neg. control</td>
<td>Pos.</td>
<td></td>
<td>RHDV</td>
<td>12800 ++ + + + + +</td>
<td>ND Neg.</td>
</tr>
<tr>
<td>Mouse sera</td>
<td>3 x v.</td>
<td></td>
<td>Negative</td>
<td></td>
<td>ND Neg. Neg.</td>
</tr>
<tr>
<td>MAbs (ascitic fluid)</td>
<td>7/6 mg/ml</td>
<td>E9</td>
<td>RHDV</td>
<td>819200 + + + +</td>
<td>ND Neg.</td>
</tr>
<tr>
<td>2/65 mg/ml</td>
<td>C3</td>
<td>RHDV</td>
<td>102400 + + +</td>
<td>ND Neg.</td>
<td></td>
</tr>
<tr>
<td>3/77 mg/ml</td>
<td>F2</td>
<td>RHDV</td>
<td>204800 + + +</td>
<td>ND Neg.</td>
<td></td>
</tr>
<tr>
<td>1/86 mg/ml</td>
<td>E7/D10</td>
<td>RHDV</td>
<td>204800 + + +</td>
<td>ND Neg.</td>
<td></td>
</tr>
<tr>
<td>5/4 mg/ml</td>
<td>E7/C3</td>
<td>RHDV</td>
<td>204800 + + +</td>
<td>ND Neg.</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>1G9</td>
<td>FCV</td>
<td>Neg.</td>
<td>ND Neg.</td>
<td>ND Neg. Neg.</td>
</tr>
</tbody>
</table>

* Infection (i.), challenge (chall.), vaccination (v.).
† IP, Direct and indirect IP demonstration of the virus in infected cells.
‡ The symbols ‘-’ and ‘+++’ represent negative and strongly positive reactions, respectively.
§ ND, Not determined.
Fig. 2 and Fig. 3. SDS-PAGE of RHDV (R, Fig. 2) and FCV (F, Fig. 3) fractionated under reducing conditions in a 10% gel. One part of each gel (a), containing the Mr standards (lane S) or the virus (lanes R and F), was stained with silver. Other parts of the gels (b), containing RHDV (Fig. 2) and FCV (Fig. 3) only, were examined by WB. Strips of NC membrane in Fig. 2 and 3 were incubated with the same rabbit (lanes 1 to 4), or mouse (lanes 5 to 12) samples. Rabbit hyperimmune anti-RHDV serum 7/88/4 (lanes 1), Chinese RHDV-positive rabbit serum (lanes 2), negative rabbit serum (lanes 3), control without rabbit serum (lanes 4), RHDV MAb E9 (lanes 5), RHDV MAb C3 (lanes 6), RHDV MAb F2 (lanes 7), MAb RHDV E7/D10 (lanes 8), MAb RHDV E7/C3 (lanes 9), FCV MAb 1G9 (lanes 10), negative mouse serum (lanes 11), control without mouse serum or ascitic fluid (lanes 12). Arrowheads show the major viral antigens on the silver-stained gels. Further details are given in Table 1.

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Fig. 3. SDS-PAGE of RHDV (R, Fig. 2) and FCV (F, Fig. 3) fractionated under reducing conditions in a 10% gel. One part of each gel (a), containing the Mr standards (lane S) or the virus (lanes R and F), was stained with silver. Other parts of the gels (b), containing RHDV (Fig. 2) and FCV (Fig. 3) only, were examined by WB. Strips of NC membrane in Fig. 2 and 3 were incubated with the same rabbit (lanes 1 to 4), or mouse (lanes 5 to 12) samples. Rabbit hyperimmune anti-RHDV serum 7/88/4 (lanes 1), Chinese RHDV-positive rabbit serum (lanes 2), negative rabbit serum (lanes 3), control without rabbit serum (lanes 4), RHDV MAb E9 (lanes 5), RHDV MAb C3 (lanes 6), RHDV MAb F2 (lanes 7), MAb RHDV E7/D10 (lanes 8), MAb RHDV E7/C3 (lanes 9), FCV MAb 1G9 (lanes 10), negative mouse serum (lanes 11), control without mouse serum or ascitic fluid (lanes 12). Arrowheads show the major viral antigens on the silver-stained gels. Further details are given in Table 1.

conjugated SwAMoIgG diluted 1:1000 and 1:100 to 1:200, respectively, were used in routine examinations. The results of incubation of positive control smears (impressions, sections) with the negative mouse serum, or FCV MAb 1G9 were uniformly negative (Table 1). Identical results were obtained by the direct IP test.

FCV was demonstrable in the CRFK cell line 6 h after infection with FCV 9 by the indirect IP and IF tests only in cultures incubated with FCV MAb 1G9 (Fig. 5). All cultures incubated with RHDV-positive rabbit sera or RHDV MAbs were negative (Table 1).

Diffusely stained cells in various stages of degeneration, as well as freshly infected cells with pronounced positive reactions in the form of granules or a ring in the cytoplasm only (Fig. 4 and 5), were seen in the IP test, which was more suitable for visual evaluation than IF.

Discussion

Five clones of hybridomas, producing MAbs to RHDV, were prepared and tested. Some of the MAbs reacted with the dominant RHDV proteins, M, 61K and 38K, in WB analysis, but others did not. However, all MAbs reacted intensely and highly specifically with native RHDV proteins, as demonstrated by high ELISA antibody titres in all samples of ascitic fluid, as well as by
their suitability for IP and IF demonstration of the virus in tissues of rabbits dying after natural or experimental infections. Therefore, we suppose the MAbs that were non-reactive with the dominant proteins in WB react with antigenic structures which are damaged during the treatment of the virus for PAGE under reducing conditions.

The ELISA technique described earlier (Rodák et al., 1990) proved fully suitable for the examination of hybridoma culture media. The possibility of using HIT for this purpose is rather unlikely. ELISA detected very low concentrations of antibodies, released into culture media by several tens of hybridoma cells only, even during first screening tests.

Cross reactions of RHDV MAbs and FCV MAbs with the dominant proteins of the other virus were observed neither in WB, nor in IP or IF tests. Antigenic differences between the two viruses were also confirmed by the non-reactivity of convalescent rabbit sera with the FCV antigen. Cross-reactions were observed only when sera of vaccinated rabbits and the FCV antigen were used as components in the ELISA (Table 1). However these sera did not react with the FCV antigen in WB, IP or IF tests. Positive reactions of the sera are therefore interpreted as the result of a reaction of non-specific antibodies with traces of cell and protein antigens present in the incompletely purified FCV antigen. The correct classification of RHDV has been complicated by its haemagglutinating activity, failure to propagate in vitro and by a number of conflicting results obtained by different authors (Liu et al., 1984; Cao et al., 1986; Shen et al., 1986; Wei et al., 1987; Xu et al., 1988; Ohlinger et al., 1989; Šmid et al., 1989). The causal agent of RHDV has most often been classified as a parvo-, picorna-, or calicivirus.

Its ultrastructure (Šmid et al., 1989; Ohlinger et al., 1989; Valiček et al., 1990) and the presence and size of the dominant protein (61K) classes RHDV with caliciviruses. Such classification is supported by the finding that both RHDV and FCV contain dominant (capsid) proteins with very similar M, values (60K to 61K and 62K to 64K), typical for caliciviruses (Carter et al., 1989; Capucci et al., 1990; Rodák et al., 1990). Moreover, using IP and IF tests both RHDV and FCV were detectable in only the cytoplasm of freshly infected cells, whereas parvovirus is located in the nucleus.

The suitability of MAbs for the IP and IF diagnosis of RHDV was confirmed by the results from examinations of tissue samples collected from rabbits dying after natural or experimental infections. The specificity of both methods was fully confirmed by electron microscopic findings. Routine use of RHDV MAbs for IP and IF diagnosis of this dangerous rabbit disease can be highly recommended. Both techniques replace HT as the only method commonly used for virus demonstration, being simpler and possessing a much higher sensitivity and specificity. The use of electron microscopy will remain limited to laboratories possessing the necessary equipment and qualified staff.

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