Bulgarian Journal of Veterinary Medicine (2009), **12**, No 1, 43–53

HETEROPHIL EMPERIPOLESIS IN RABBIT HAEMORRHAGIC DISEASE

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Summary

Alexandrov, M., R. Peshev, S. Lasarova, L. Doumanova, A. Tchorbanov & R. Bostandjieva, 2009. Heterophil emperipolesis in rabbit haemorrhagic disease. *Bulg. J. Vet. Med.*, **12**, No 1, 43–53.

Liver and spleen obtained from rabbits deceased after inoculation with rabbit haemorrhagic disease virus (RHDV) were studied by means of light, transmission electron and immunoelectron microscopy. Severe damages located within the nuclei and cytoplasm of many periportal hepatocytes and splenic macrophages were detected. However, the most prominent pathological lesions were multifocal liver necroses abundantly infiltrated with heterophil granulocytes, as well as heterophils found throughout the red pulp of the spleen. Ultrastructurally, unique cell interactions were established. The cytoplasm of many hepatocytes/splenic macrophages was invaded with heterophils. Therefore, the term emperipolesis (penetration of heterophils toward the hepatocytes/splenic macrophages) was preferred for outlining these lesions. It was assessed that the observed emperipolesis may play a key role in the rabbit haemorrhagic disease pathology, as well as it could be the main reason for the development of smaller RHDV particles found by electron microscopy in the RHDV-positive specimens.

Key words: electron microscopy, emperipolesis, immunoelectron microscopy, rabbit haemorrhagic disease, rabbit haemorrhagic disease virus

INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious illness with lethal outcome in adult rabbits. On the contrary, juvenile animals up to eight weeks of age are not susceptible (Peshev *et al.*, 1989; Gregg *et al.*, 1991; Kuttin *et al.*, 1991). The causative agent – the rabbit haemorrhagic disease virus (RHDV) was assigned to the Caliciviridae family, genus *Lagovirus* (Ohlinger *et al.*, 1990; 1991; Koopmans *et al.*, 2005). It has been established that the main target organs of the RHDV are the liver and spleen (Carrasco *et al.*, 1991; Alexandrov *et al.*, 1992; Park & Itakura, 1992; Mikami *et al.*, 1999; Prieto *et al.*, 2000; Kimura *et al.*, 2001). The main lesions consist of a haemorrhagic syndrome and severe necrotizing hepatitis where necrotic foci are markedly infiltrated with heterophil leukocytes (Belemezov *et al.*, 1989; Marcato *et al.*, 1990; Kuttin *et al.*, 1991; Fuchs & Weis-

senbock, 1992). In some studies it was also found that the liver infiltrates had been made up mostly of heterophils located near hepatocytes or within hepatocytes showing severe cellular damage (Park *et al.*, 1992; Ferreira *et al.*, 2005). Similar cell interactions described as emperipolesis were discovered in various other pathological conditions where living or damaged cells had been invaded by other cells (Harb *et al.,* 1974; Tanaka *et al.,* 1997; Caruso *et al.,* 2002; Gómez-Villamandos *et al.,* 2003; Centurione *et al.,* 2004; Mehraein *et al.,* 2006; Hoshino *et al.,* 2008). Furthermore, it was pointed out that organ homogenate supernatants, prepared from rabbits with RHD or purified RHDV preparations, in addition to the typical calicivirus particles with a diameter of 32 to 40 nm contained virions of a distinctly smaller diameter (25−29 nm) with a featureless surface (Capucci *et al.,* 1991; Alexandrov *et al.,* 1992; Moussa *et al.,* 1992; Granzow *et al.,* 1996; Barbieri *et al.,* 1997).

In the light and electron microscopy examinations over a period of several years on rabbits infected with RHDV with lethal outcome used for vaccine production, we have detected a number of animals showing the above pointed pathological features. Analyzing the morphological features we assumed that the emperipolesis may play a key role in some aspects of the pathogenesis of RHD and the variability of RHDV particles. Therefore, the aim of the present studies was to give more a detailed description of these findings.

MATERIALS AND METHODS

Experimental animals

Nineteen nonimmune adult outbred rabbits (*Oryctolagus cuniculus*) used for anti RHD inactivated vaccine production were selected for this study. Each animal was intramuscularly inoculated with a rabbit organ suspension that had 2^{12} haemagglutination units of RHDV (isolate Slivnitsa/88). Tissue samples were taken from the liver and spleen immediately after the death of each animal (1 to 5 days post infection) as previously described (Peshev *et al.,* 1998).

Rabbit anti RHDV antibodies (RbARHDV-Abs)

Hyperimmune rabbit anti RHDV sera were prepared and rabbit anti RHDV antibodies (RbARHDV-Abs) were purified by means of DEAE Affi-Gel Blue (Bio Rad) ion exchange chromatography as described earlier (Alexandrov *et al.,* 1992).

Haemagglutination tests

Haemagglutination was performed according to Peshev *et al.* (1998) in microtitration multi-well plate with 96 U-shaped wells (Linbro/Titertek, Flow Laboratories, McLean, Virginia USA) at room temperature. Briefly, the antigen was serially two-fold diluted with 0.05M NaH_2PO_4/ Na_2HPO_4, 0.15M NaCl (pH 7.2) and equal amounts of 0.5% suspension of human erythrocytes type "O" were added. The reactions were admitted after deposition of erythrocytes in control rows as button. The haemagglutination titre was taken as the reciprocal of the highest dilution producing complete agglutination of erythrocytes.

Histology

Tissue samples from the liver and spleen of all rabbits were fixed for 48 h in 4% buffered formaldehyde, embedded in paraffin, prepared in 5 to 8 μm sections and stained with hematoxylin-eosin.

Electron microscopy of ultrathin sections

Small organ pieces from the liver and spleen of all rabbits were fixed for 2- h in 4% glutaraldehyde in 0.2 M cacodylate buffer at 4 °C, pH 7.2, post fixed for 2 h in 2% OsO_4, dehydrated in graded alcohols and through propylene oxide, propylene oxide-resin embedded in Durcupan (Fluka). Pyramids with 4 mm^2 surface were formed and semithin sections (0.5 up to 1 μm thick) were prepared with a glass knife on a Reichert ultratome. The sections were mounted on glass slides, stained with 1% toluidine blue in 1% borax and examined under a light microscope. Areas with inflammatory foci were selected and pyramids were additionally trimmed for ultratomy. Yellow-gold ultrathin sections (70−100 nm thick) were prepared and mounted on 400 mesh copper grids and stained with saturated solution of uranyl acetate followed by lead citrate according to the standard technique.

Solid phase electron microscopy

Liver and spleen pieces from all mortally diseased rabbits were frozen and thawed thrice and prepared as 10% homogenates in phosphate buffered saline (PBS), pH 7.2. The latter were clarified by centrifugation at 20,000×*g* for 20 min and the supernatants were layered onto 30% (w/w) sucrose solution and concentrated by ultracentrifugation (109,000×*g*, 10 h, 4°C). The pellets were resuspended in PBS, clarified at 5,000×*g* for 20 min and pelleted again at 109,000×*g* for 2.5 h. The final virus containing pellets were resuspended in a minimal volume of bidistilled water and analyzed by the transmission electron microscopy after negative staining with 1% uranyl acetate, pH 4.2 as described earlier (Alexandrov *et al.*, 1993).

Solid phase immunoelectron microscopy

The surface of formvar and carbon coated 400 mesh copper grids were sensitized for 30 min with RbARHDV-Abs by floating the grids on a 50 μL drop of antibodies. Then they were washed on 3 consecutive drops of PBS and floated for 30 min on native virus suspensions (supernatants of centrifuged at 3,000×*g* liver homogenates 10% W/V). After three washings on 50 μL drops of PBS, they were negatively stained as described above.

All electron microscopy examinations were carried out on an electron microscope JEOL 1200 EX at an accelerating voltage of 80 kV and an instrumental magnification of 2,000–75,000×. Electron micrographs were taken on Kodak EM Film 4489, 6½×9 cm. To evaluate the images, the negatives were scanned directly on a HP Scanjet 4890 scanner at a resolution of 600 dpi using the "SCAN FILM" option.

Morphometric analysis

The diameter of the viral particles was determined using the image analyzing computer program Image-Pro Plus version 6 (Media cybernetics). The electron micrographs were scanned directly as negatives on a HP Scanjet 4890 scanner at 600 dpi resolution using the "SCAN FILM" option. The diameter of 200 typical, as well as 200 core-like virus particles were separately measured using the automatically obtained calibration line on the right side of the electron micrographs to calibrate the measurements.

RESULTS

Histopathology

All rabbits selected in this study were with multifocal necrotic foci in the liver

located mainly in the periportal and intermediate acinar zones (zones 1 and 2). They were abundantly infiltrated with heterophils (Fig. 1 A-C). The heterophilic exudation was also present in the sinusoids. Karyolysis, pyknosis, bile pigment deposition, apoptotic figures, cytoplasmic swelling of hepatocytes as well as totally lytic hepatocytes were a usual finding.

The spleen of some rabbits appeared congested with follicular karyorrhexis of some lymphocytes but in eight rabbits it was abundantly infiltrated with heterophils in the red pulp as well. The light microscopy of the semithin sections of the liver and spleen tissues revealed the above-mentioned damages and was the basis for targeted ultratomy (Fig. 1 C).

Ultrastructural findings

The present ultrastructural investigations revealed that many hepatocytes were strongly damaged. There was an extensive disorganization of the hepatic architecture. Cell shrinkage, variable nuclear changes and disruption of plasma membranes were a common finding. Some nuclei were shrunk, some swollen but in others, nuclear membrane blebbing, chromatin cleavage, chromatin condensation, formation of pyknotic bodies of condensed chromatin or irregular rearragement of chromatin were also observed. Single nuclei were with abundance of interchromatin granules. Nuclei with lytic chromatin in totally disintegrated cells but with discernible membranes were also observed. Many of them had vacuoles of

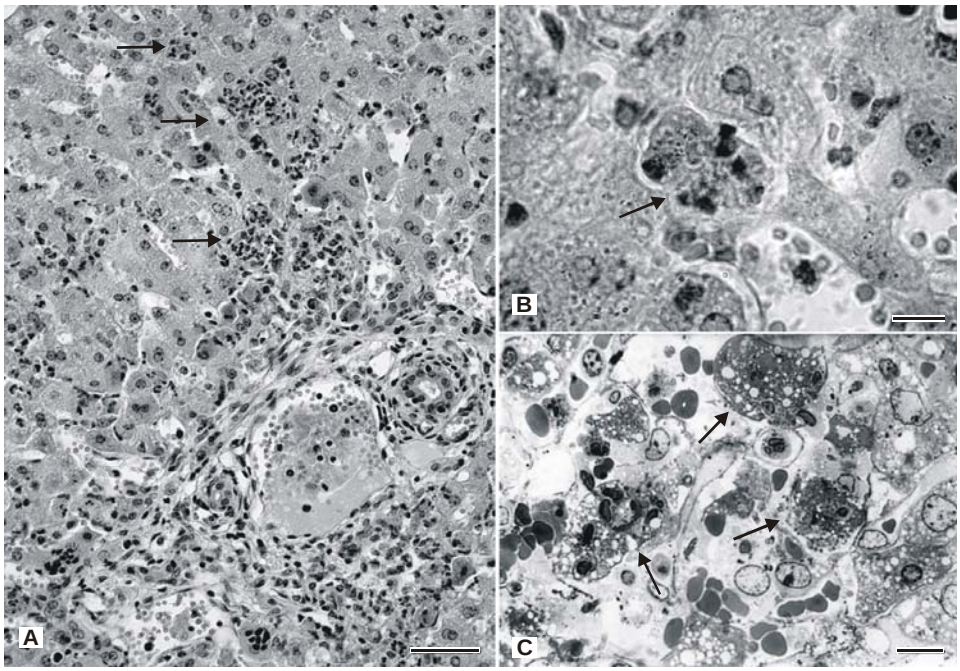

Fig. 1. Histological features of a rabbit liver at the 49th h post inoculation (PI) with RHDV. A-C. Multiple areas (arrows) of necrosis associated with influx of heterophils; (B, C. Some of hepatocytes (arrows) are abundantly invaded by heterophils; Paraffin sections stained with hematoxylin-eosin (A, B) and semithin section stained with 1% toluidine blue (C); bars: A = 50 µm; B, C = 10 µm.

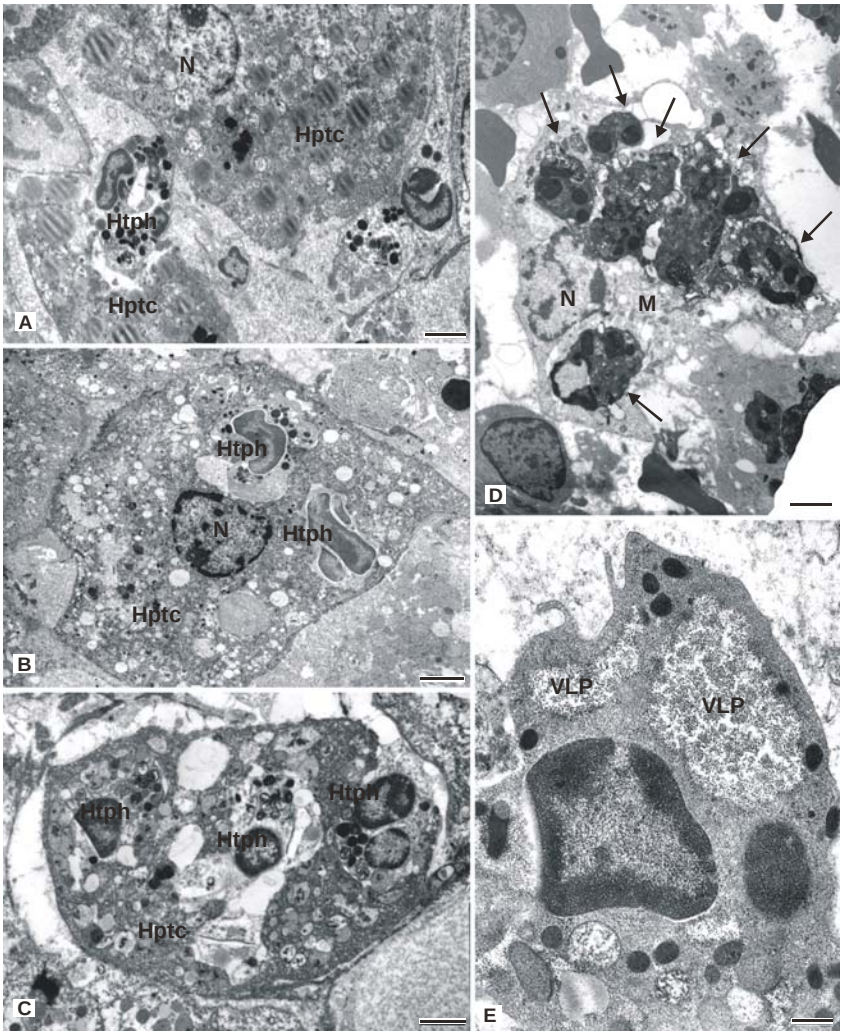

Fig. 2. Electron micrographs of ultrathin sections from rabbit livers and spleen infected with RHDV. A. Liver at the 26th h PI. The right heterophil leukocyte (*Htph*) is attached to the cell surface of the right-sided upper hepatocyte (*Hptc*) and the left Htph is passing through the membrane of the left sided hepatocyte below. Both hepatocytes contain many lipid droplets; B. Emperipolesis – two heterophil leukocytes within the vacuolated cytoplasm of a hepatocyte in a rabbit liver at the 49th h PI. C. Emperipolesis – three heterophil leukocytes are inside the cytoplasm of a heavily damaged hepatocyte in a rabbit liver at the 74th h PI; D. At least six heterophil leukocytes (arrows) are inside the cytoplasm of a strongly damaged perifollicular macrophage (*M*) in a rabbit spleen at the 72nd h PI, where the macrophageal nucleus (*N*) could be used as a cell identification marker; E. Various-sized vacuoles containing a number of oval or hexagonal electron dense granules within the cytoplasm of a heterophil in a rabbit spleen at the 72nd h PI. These granules can be perceived as glycogen or RHDV like particles (*VLP*). Uranyl acetate-lead citrate staining. Bars: A-D = 2 μm, C = 500 nm.

different shape and size at the periphery. In binucleated hepatocytes, both nuclei were with similar or absolutely diverse changes. Numerous electron-dense material, electron dense granules or electron-lucent particles were seen in the Golgi vacuoles. Some hepatocytes contained helical polyribosomes or ribosome crystals. Polyribosomes were disaggregated but were closely attached to the distended rough endoplasmic reticulum which contained fine granules. The cytoplasm of many necrotic hepatocytes contained numerous membrane-bound vesicles and vacuoles which appeared empty or were filled with electron-dense materials. Mitochondria were swollen, with a loss of mitochondrial dense granules or totally disintegrated. Different in size lipid granules were detected in many cells. Contrary to the described above features, in some livers, the cytoplasmic organelles of many parenchymal hepatocytes were transformed to moderate electron dense masses of an irregular shape and filamentous structures so that the rearranged cytoplasm sometimes resembled a peculiar point lace. Besides, a lot of periportally and midzonally located hepatic foci were abundantly infiltrated with heterophils and heterophil emperipolesis within the hepatocytes was the most prominent pathological feature there. Heterophils were found in sinusoids, lying free within the cell detritus, attached or passing through the cytoplasmic membrane of a hepatocyte, as well as often established within the cytoplasm of hepatocytes (Fig. 2A and B). In some foci many heterophils were situated totally within the cytoplasm of the parenchymal liver cells (emperipolesis), where up to eight heterophils of a hepatocyte could be counted (Fig. 2C). Various morphological features were found among the heterophils. They were irregular in shape with segmented and lobed nucleus. There were many pseudopodes on the heterophil surface. In some heterophil nuclei, projections were present arising from the nuclear surface. Various-sized vacuoles containing a number of oval or hexagonal electron dense granules with a mean diameter of 29.84606 nm (min 23.98373, max. 37.85785, std. dev. 2.879875) were regularly observed within the cytoplasm of heterophils. These granules could be perceived as glycogen or RHDV like particles (Fig. 2E). Both primary and secondary lysosomal granules were presented within the cytoplasm and were used as heterophil identifying markers.

Ultrastructural changes in the spleens were not constant findings. Only in 6 rabbits the heterophilic emperipolesis was detected within macrophages located near the white pulp. Single splenic macrophages like some hepatocytes were invaded with heterophils (Fig. 2D). The heterophils in the macrophages and those in the red pulp were of a morphological appearance undistinguishable from the heterophils infiltrating the liver.

Detection of viral particles by electron microscopy

The results of hemagglutination assays indicated the presence of RHDV in the liver and spleen homogenates. All samples contained completely formed virus particles (58.020477%) of a mean diameter 37.69521 nm (min. 31.57093, max. 44.48759, std. dev. 2.328655). They were with cup-shaped depression borders and surface indentations typical of the classical RHDV appearance. Among them smaller virions (41.979523%) with icosahedral symmetry and a mean diameter of 28.37952 nm (min. 22.5521, max. 33.66054, std. dev. 1.975249), as well as scattered ferritin like particles were also seen (Fig. 3A).

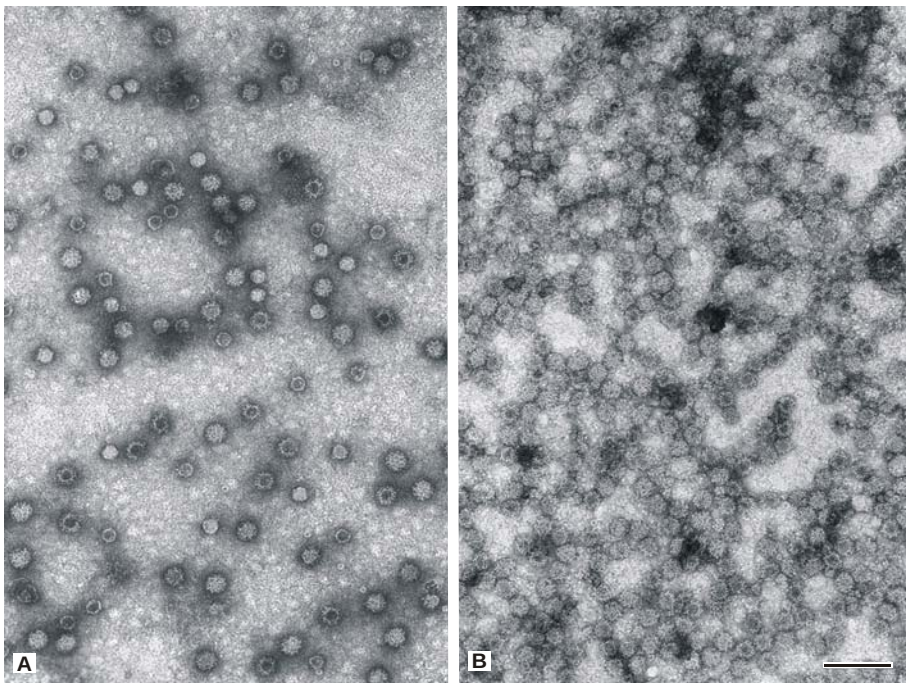

Fig. 3. A. Purified RHDV from liver homogenate of a rabbit deceased at the 72nd h PI. Full and empty classical and core like virus particles, as well as ferritin-like bodies are present; B. Electron micrograph of RHDV detected in the same homogenate by means of immunoelectron microscopy - only classical virus particles are visible. Negative staining with 1% uranyl acetate. Bar = 100 nm.

When the immunoelectron microscopy was used, only completely formed RHDV particles were abundantly enriched on the grid surface sensitized with RbARHDV-Abs. It seemed that the smaller virions were not present among the observed viral particles (Fig. 3B).

DISCUSSION

Many aspects of the pathological features of rabbits deceased of RHD observed in the present studies were described by other researchers (Belemezov *et al.,* 1989; Marcato *et al.,* 1990; Kuttin *et al.,* 1991; Park *et al.,* 1992; Alonso *et al.,* 1998; Ferreira *et al.,* 2005). However, attention was paid to the cell interaction within the inflammatory foci of liver and spleen of rabbits with a fatal course of the infection in a small number of studies only (Park *et al.*, 1992; Ferreira *et al.*, 2005). The present investigations therefore clearly indicate that interesting cell interactions were present in the RHDV-infected rabbit liver and spleen. The last were manifested as heterophilic invasion in the cytoplasm of hepatocytes and splenic macrophages. The term emperipolesis (penetration of heterophils toward the hepatocytes) was therefore preferred for outlining these lesions. When the ultrastructural studies were conducted the dynamics of the observed emperipolesis could be outlined as followed: Heterophils were found in hepatic sinusoids, lying free in necroses,

adjacent to the hepatocytes/splenic macrophages, passing through hepatocytes/splenic macrophageal plasma membrane and finally often established in the cytoplasm of hepatocytes/splenic macrophages. Based on these findings and simultaneously discovering the heterogeneity of RHDV particles it can be presumed that emperipolesis may play a key role not only in the systemic lesions but on the development of smaller virus particles as well. Besides, the target cells of the RHDV (hepatocytes and macrophages) should also be at a certain stage of differentiation because according to the current knowledge, rabbits cannot be mortally diseased with RHD until they reach the age of about two months (Gregg *et al.*, 1991; Kuttin *et al.*, 1991; Ferreira *et al.*, 2004; Ferreira *et al.*, 2005). However, it is not yet clear why heterophils invade necrotic hepatocytes and no definitive explanation was given in other studies where emperipolesis of neutrophils and lymphocytes toward other cells was described (Tanaka & Fujita, 1997; Pinho *et al.*, 2001; Gómez-Villamandos *et al.*, 2003). Emperipolesis in some studies was explained to be a mechanism which improves cell survival, and helps prevent apoptosis of cells within the host cell (Lindhout *et al.*, 1995; Tsunoda *et al.*, 1988; Tsunoda *et al.*, 1992) or was associated with cytotoxicity to the host cell (Fujinami *et al.*, 1981; Tobe, 1982). In the meanwhile, the most similar pathological changes corresponding to the findings in the present studies were found in the liver of mice, experimentally infected with the encephalomyocarditis virus (Harb *et al.*, 1974). Therefore, it is very likely that emperipolesis is frequently involved in many hepatic inflammatory diseases and other pathological conditions but that it is often missed when only routine light microscopy analyses are performed. According to our findings it could be also presumed that heterophil-hepatocyte emperipolesis is associated with RHDV infection when the virus propagation is at a higher level in infected cells and plays an important role in the removal of the viral particles or parts of damaged hepatocytes and splenic macrophages, as well as that it is responsible for the formation of smaller viral particles following digestion of the standard RHDV particles in heterophil phagosomes as we believe. However, it is difficult to support this opinion because RHDV particles in our ultrastructural preparation were unclear and could be mistaken for the glycogen granules characteristic for rabbit hepatocytes and heterophils (Bozzola & Russell, 1999). Independently of that in our previous studies, using negative staining techniques and transmission electron microscopy we did not or rarely could find smaller RHDV particles in adult rabbits analyzed up to the 24th hour after the challenge (Alexandrov *et al.,* 1993). Although the heterogeneity of the RHDV particles could be easily detected in negative staining preparations, some studies performed to explain these findings led to controversial conclusions. Granzow *et al.* (1996) assume that the smaller core-like particles are not the result of a proteolytic digestion but arise from a truncated RHDV genome or defective expression. Another assumption is that the genesis of the particles is due to a degrading process that is probably the consequence of the physiological clearance of the RHDV-IgM immuno-complex formed in large amounts at the beginning of the humoral response and that the identification of these smaller particles in the rabbit liver can be considered a marker of the subacute/chronic form of RHD that usually evolves between days 4 and 8 post-infection and is

followed either by the death of the rabbit or, more often, by its recovery (Barbieri *et al.,* 1997). The present studies of ours could not confirm the last statement because the corelike RHDV particles were present even in livers obtained from the rabbits deceased between hours 24−48 post-infection, when the formation of RHDV-IgM immuno-complex is hardly possible but the heterophil-hepatocyte emperipolesis was detected. On the other hand, when the immunoelectron microscopy was used, the grids sensitized with RbARHDV-Abs were abundantly enriched with completely formed RHDV particles only. Therefore, it seemed that the smaller virions which had never been observed in our immunoelectron microscopy preparations were less or not at all antigenically responsible and no definitive explanation could be given for that at this time. Further studies are therefore necessary to clarify the proper biological mechanisms of observed unusual and interesting cell interactions where the parenchymal liver nonphagocytic cell is invaded with the smaller heterophils. Also, the extent heterophil-hepatocyte emperopolesis associates with the heterogeny of the RHDV particles or plays an important role in the pathogenesis of the RHD should be made clear.

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Paper received 07.01.2009; accepted for publication 02.02.2009

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