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Apoptosis of peripheral blood leucocytes in rabbits infected with different strains of rabbit haemorrhagic disease virus

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The pathogenicity of RHDV (rabbit haemorrhagic disease virus) is mainly associated with its affinity to blood vessels, with causing disseminated intravascular coagulations (DIC), and with the stimulation of the host immune system. Moreover, there are implications suggesting that apoptosis may be a pivotal process in understanding the basis of viral haemorrhagic disease in rabbits — a serious infectious disease causing mortality to wild and domestic rabbits. The aim of this study is to evaluate, by means of flow cytometry, the dynamics of apoptosis in peripheral blood granulocytes and lymphocytes in rabbits experimentally infected with seven different strains of RHDV and so-called antigenic variants of RHDV denominated as RHDVa, i.e.: Hungarian 24V/89, 1447V/96, 72V/2003; Austrian 01-04, 237/04, V-412 and French 05-01. The results showed that all of the RHDV and RHDVa strains cause an increase in the number of apoptotic cells throughout the infection, which might indicate the need for further analysis of the importance of this process.

Key words: RHDV, RHDVa, apoptosis, granulocytes, lymphocytes

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INTRODUCTION

The mechanism of pathogenic action of rabbit haemorrhagic disease virus (RHDV) is mainly associated with its affinity to blood vessels, in which it has a damaging effect on the endothelium, and causes disseminated intravascular coagulation (DIC) (Ueda *et al.,* 1992). It has also been shown that RHDV infection results in the stimulation of host immune system (Tokarz-Deptuła, 2009; Niedźwiedzka-Rystwej & Deptuła, 2010) and induction of apoptosis in hepatocytes, in which programmed cell death serves as the main tool for viral replication (Alonso *et al.,* 1998; Jung *et al.,* 2000; San-Miguel *et al.,* 2006; Ni *et al.,* 2009; Garcia-Lastra *et al.,* 2010; Marques *et al.,* 2010; Tuñón *et al.,* 2011; Niedźwiedzka-Rystwej & Deptuła, 2012). The role of apoptosis in RHD virus infection was studied on rabbits infected with RHDV strains of an unknown origin (Table 1). However, some investigations were performed using the strains with defined places of isolation (Alonso *et al.,* 1998; Niedźwiedzka-Rystwej & Deptuła, 2012; Niedźwiedzka-Rystwej *et al.,* 2013). The course of these processes was assessed post mortem in experimentally infected rabbits (a static model), by means of histopathological, spectrofluorimetric and flow cytometric methods. The only data indicating dynamic changes in the host organism after infection with the RHD virus were presented in Polish studies (Niedźwiedzka-Rystwej & Deptuła, 20010; 2012). In all cited investigations apoptosis was shown to be induced not only in hepatocytes, but also in macrophages (mainly in the spleen and lymph nodes), monocytes, endothelial cells, lymphocytes and granulocytes of infected rabbits, which indicates its significant role during the RHDV infection.

The aim of the current study was to evaluate the extent of apoptosis in peripheral blood granulocytes and lymphocytes in rabbits experimentally infected with seven, not yet investigated, haemaglutinating strains of RHDV and RHDVa. Presented research may explain some of the processes involved in the pathogenic action of the RHD virus, as well as the pathogenesis of rabbit plague.

MATERIALS AND METHODS

Animals used in the study. The study included 140 mixed-breed rabbits of both sexes weighing between 3.2 and 4.2 kg. The rabbits, marked as conventional animals, were purchased from a licensed breeding farm and maintained under constant supervision of veterinary and animal husbandry staff (Anon, 1987). During the experiment, the animals were housed in the vivarium of the Microbiology and Immunology Departments at the Faculty of Biology, University of Szczecin. Parameters such as lighting, ventilation, and the size of cages were based on the standards recommended in Poland and elaborated in accordance with the European Union directive (Anon 2010). After transportation to the vivarium, the animals were subjected to a two-week adaptation period. The rabbits were fed with full-portion rabbit feed (Motycz, Poland) and provided with unlimited access to water.

Viruses used in the study. The rabbits were infected with RHDV strains isolated in different time periods from animals that died naturally, and were infected with the following haemaglutinating strains: Hungarian 24V/89 isolated in 1989, Hungarian 1447 V/96 isolated in 1996, haemaglutinating antigenic variant 72V/2003 isolated in 2003, Austrian 01-04 isolated in 2004, Austrian 237/04 isolated in 2004, V-412 isolated in 1989, and French 05-01 isolated in 2005. The following strains: 24V/89, 1447 V/96, 72V/2003 were obtained from

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Abbreviations: DIC, disseminated intravascular coagulation; RHDV, rabbit haemorrhagic disease virus

Professor Istuan Kiss from the Central Veterinary Institute, Institute of Debrecen, Hungary. The strains: 01-04, 237/04, V-412 were donated by Professor Lubomir Valiček from the Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Brno, Czech Republic, while the strain 05-01 was received thanks to dr. Ghislane Le Gall-Recule from AFSSA – the French Food Safety Agency. Liver homogenates of rabbits infected with these strains were used for experimental infection. Livers were collected from the experimentally infected rabbits after death, and 20% of liver homogenates were prepared and purified by centrifugation at 1500 × *g* followed by a treatment with 10% chloroform for 60 min and additional centrifugation. The liver homogenate samples were then suspended in glycerol at a 1:1 ratio (Niedźwiedzka-Rystwej & Deptula, 2010). All of the RHDV preparations contained the same relative density (1.34 g/dm^3), determined by CsCl density-gradient centrifugation. The animals were divided into groups of 10, and each group was infected with one of the seven RHDV strains by intramuscular injection in the lower limb muscle with a dose of RHDV antigen suspended in 1 ml glycerol. Simultaneously, control groups were created (10 animals per group), correspondingly to the existing experimental groups, in which animals were injected with a placebo (1 ml glycerol). Blood samples were collected from the peripheral ear veins of all the rabbits (control and experimental) at time 0 (before RHDV infection) and at time points 4, 8, 12, 24, and 36 h after infection. At that time the clinical symptoms were also monitored, including the mortality rate of each group. Additionally, the parameters of the vivarium (temperature and humidity) were controlled every 24 h (at 8.00 am every morning).

Flow cytometric measure of apoptosis. Apoptosis was assessed in each sample using an ApoFluor®Green Caspase Kit (MP Biomedicals, USA) and a FACScan flow cytometer (Becton Dickinson, USA), equipped with Cell Quest software (USA). The total activity of caspases: 1, 3, 4, 5,6, 7, 8 and 9 (MP Biomedicals, USA) in peripheral blood granulocytes and lymphocytes of rabbits was determined and expressed as percentage of apoptotic cells in these populations. The samples were prepared by centrifugation of heparinised blood for 5 min at 400 × *g*. After centrifugation, 300 µl of each supernatant was placed in a fresh eppendorf tube, 10 µl of ApoFluor®Green fluorescent dye was added, and the samples were incubated for 60 min at 37°C in the presence of 5% CO_2, with gentle agitation every 20 min. Excess fluorescent dye was then removed by washing the cells with 2 ml of washing buffer, and the samples were centrifuged for 5 min at 400 × *g* at room temperature. The supernatants were then discarded, and the centrifugation step was repeated. The ApoFluor®Green-dyed cell pellets were then incubated with 400 µl washing buffer containing 2 µl propidium iodide (PI). After a 10-minute incubation on ice, the samples were analysed by flow cytometry. The use of two fluorescent dyes with different emission spectra (green for ApoFluor®Green and red for PI) allowed for identifying the viable, apoptotic, and dead (necrotic) cell populations. The results are presented as the frequency of apoptotic cells in the total granulocyte and lymphocyte populations, differentiated on the basis of size and granularity of the cells, as well as the staining characteristics. The cells showing only ApoFluor®Green positive staining: Apo(+)PI(−), and cells positively stained with both ApoFluor®Green and PI: Apo(+)PI(+) were defined as apoptotic (Niedźwiedzka-Rystwej & Deptula, 2012; Niedźwiedzka-Rystwej *et al.,* 2013).

RESULTS AND DISCUSSION

The obtained results of dynamic changes in the number of apoptotic cells during the RHDV infection of rabbits are presented in Figs. 1 and 2. In order to com-

Table 1. Studies concerning apoptosis in rabbits infected with RHDV.

Reference	RHDV strain	Method	Time of experiment	Apoptotic cells	Time of observed changes
Alonso *et al.,* 1998	Spanish AST/89	Immunohisto-chemistry, histopatology	Animals anaesthetized in 12, 24, 40 h p.i.	Hepatocytes, macrophages(speen and lymph nodes), monocytes, endothelium	In 40 h p.i.
Garcia-Lastra *et al.,* 2010	Not defined	Caspase 3 activity (spectrofluorimetry)	Animals anaesthetized in 12, 24, 36, 48 h p.i.	Hepatocytes	In 36, 48 h p.i.
Jung *et al.,* 2000	Not defined	Immunohisto-chemistry, histopatology	Animals anaesthetized in 12, 24 h p.i.; others in 30-31 h p.i. (after death)	Hepatocytes	In 24, 30, 31 h p.i.
Ni *et al.,* 2009	Not defined	Caspase 3 activity (flow cytometry)	RK13 cells infected with RHDV	No	In 48 h p.i.
San-Miguel *et al.,* 2006	Not defined	Caspase 3 activity (spectrofluorimetry)	Animals anaesthetized in 12, 24, 36 h p.i.	Hepatocytes	In 36, 48 h p.i.
Marques *et al.,* 2010	Not defined	Annexin V activity (flow cytometry)	Animals anaesthetized in 24 h p.i.	Lymphocytes of live rand spleen	In 24 h p.i.
Tuñón *et al.,* 2011	Not defined	Caspase 3 activity (spectrofluorimetry)	Animals anaesthetized in 36 h p.i.	Hepatocytes	In 36 h p.i.
Niedźwiedzka *et al.,* 2013	Italian (BS89, Vt97, Pv97) German (Hagenow,Frankfurt,Triptis, Hartmannsdorf), English (Rainham), French (9905), Spanish (Asturias)	Caspases 1,3,4,5,6,7,8,9 activity (flow cytometry)	Animals died in 24 and 36 h p.i., blood sampling 0, 4, 8, 12, 24, 36 h p.i.	Lymphocytes and granulocytes of peripheral blood	In 4–36 h p.i., depending on strain

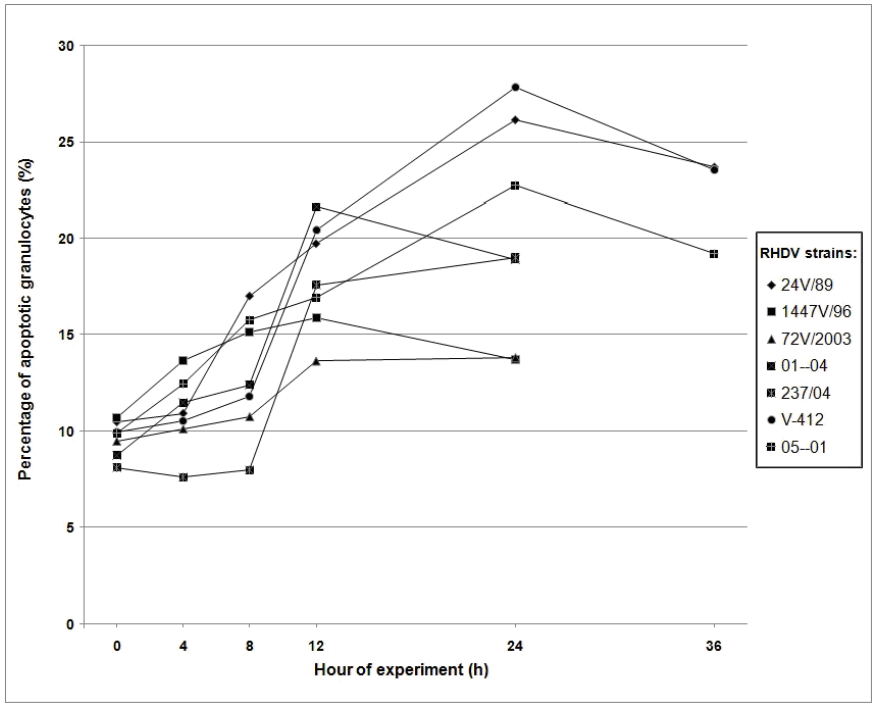

Figure 1. Dynamics of apoptotic granulocytes in rabbits experimentally infected with RHDV.

pare the data obtained in the experimental groups with the control groups, a statistical analysis was performed using the Student's *t*-test (Statistica software ver. 6.0), which results are presented in Table 2. These outcomes show statistically significant changes in the monitored parameter in the defined time points after infection (with the significance level set at $p=0.05$). Flow cytometric analysis of the percentage of apoptotic cells in the populations of granulocytes and lymphocytes in rabbits experimentally infected with the investigated haemaglutinating RHDV strains, namely: three Hungarian (24V/89, 1447 V/96, 72V/2003 — the antigenic variant of RHDV), three Austrian (01-04, 237/04, V-412) and one French (05-01), revealed that the induction of programmed cell death was observed from the 4th h after infection, and lasted until 24–36 h post infection, depending on the used strain. It should be underlined that statistically significant increases in the number of apoptotic cells, which were noted in the defined periods of time after infection (4–36 h), were more pronounced in the lymphocyte population in comparison to the granulocytes (Table 2, Figs. 1, 2).

The detailed analysis has shown that the Hungarian RHDV strain 24V/89 caused an increase in apoptosis of granulocytes at 8th, 12th, 24th, and 36th h post infection, while in case of lymphocytes these changes were observed at the 8th and 12th h. The second investigated Hungarian strain (1447 V/96) induced the apoptosis earlier, since the increased number of apoptotic granulocytes was noted in the 4th, 8th, and 12th h after infection, and an increase in the apoptotic lymphocytes was observed at 4th, 12th, and 24th h p.i. The third Hungarian strain used in this study (72V/2003), caused changes in the number of apoptotic cells only in the lymphocyte population, in the 24th h after infection. In case of the Austrian strain 01-04 changes in the percentage of apoptotic granulocytes were noted at 12th and 24th h after infection, however an earlier response was observed in lymphocytes, in the 8th, 12th, and 24th h post infection. In case of the Austrian 237/04 RHDV strain, changes in the number of granulocytes were observed 24 h post infection, while in apoptotic lymphocytes – in the 8th and 12th h after infection. The last investigated Austrian strain V-412 induced these changes at 12th, 24th, and 36th h post infection in granulocytes and lymphocytes. Finally, when rabbits were infected with the French strain 05-01,

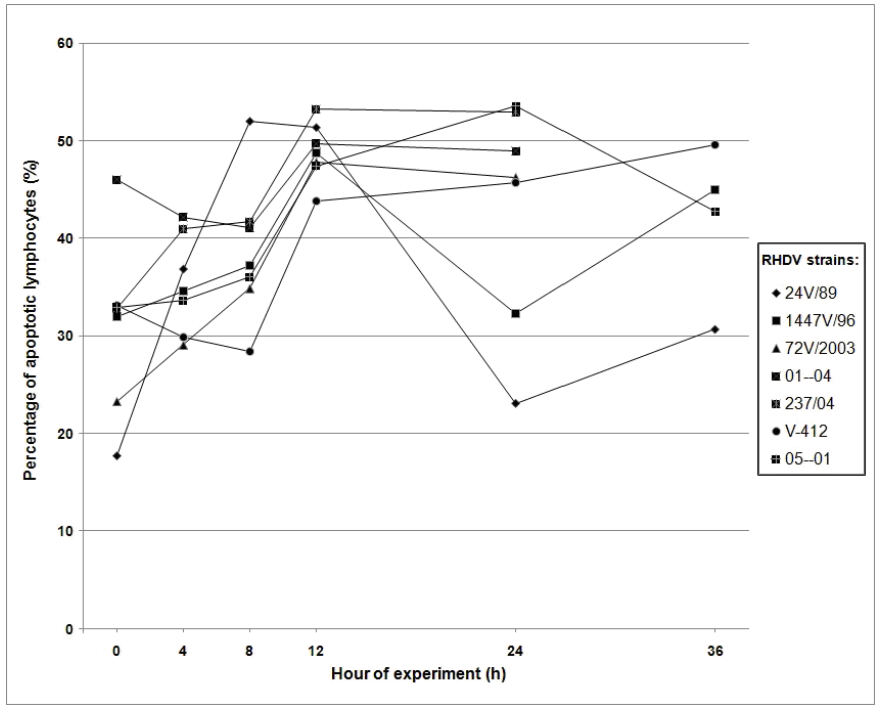

Figure 2. Dynamics of apoptotic lymphocytes in rabbits experimentally infected with RHDV.

Table 2. Percentage of apoptotic granulocytes and lymphocytes in rabbits experimentally infected with seven RHDV strains from different countries of Europe.

RHDV strain	Country of origin	Biological feature (s)	Times after infection (h) when increased percentage of apoptotic cells was detected	
			granulocytes	lymphocytes
24V/89	Hungary	HA+	8,12,24,36	8,12
1447V/96	Hungary	HA+	4,8,12	4,12,24
72V/2003	Hungary	HA+ RHDVa	none	24
01-04	Austria	HA+	12,24	8,12,24
237/04	Austria	HA+	24	8,12
V-412	Austria	HA+	12,24,36	12,24,36
05-01	France	HA+	24,36	8,12,24,36

Legend: ↑↓, statistically important increase/decrease, numbers stand for the hours of experiment; HA, haemagglutination; RHDVa, antigenic variant of RHDV

an increase in apoptotic lymphocytes was observed already in the 8th h post infection, and lasted until the end of the experiment (12th, 24th, 36th h), while the apoptosis of granulocytes was noted only in the 24th and 36th h after infection.

Results obtained in this study show that time-dependent changes in the number of apoptotic granulocytes and lymphocytes of rabbits infected with seven haemaglutinating RHDV can only be compared with previous studies (Niedźwiedzka-Rystwej & Deptuła, 2012; Niedźwiedzka-Rystwej *et al.,* 2013), in which dynamic changes in the induction of apoptosis in peripheral blood cells of rabbits experimentally infected with 10 RHD virus strains showing different biological properties were also monitored. The cited reports described the process in the following RHDV strains: haemaglutinating BS89 strain, Hagenow strain with variable haemaglutinating properties, three non-haemaglutinating strains: Rainham, Frankfurt, Asturias, and RHDVa: 3 haemaglutinating Vt97, Triptis, Hartmannsdorf, as well as 2 non-haemaglutinating Pv97, and 9905.

When we compared the presently obtained results to the effects caused by haemaglutinating RHDV strain BS89 in infected rabbits (Niedźwiedzka-Rystwej *et al.,* 2013), we noted that the highest similarity in the pattern of changes in the percentage of apoptotic granulocytes and lymphocytes was between the 237/04 and BS89 RHDV strains. The five other strains investigated in this study (24V, 1447V/96, 01-04, V-412 and 05-01) induced more intense changes (i.e. long lasting) in the number of apoptotic cells of both granulocytes and lymphocytes. Differences in the host response were also noted between the infection caused by 72V/2003 RHDVa and the previously reported data on the effect of other antigenic variants (Vt97, Triptis, Hartmannsdorf) (Niedźwiedzka-Rystwej *et al.,* 2013). The 72V/2003 variant did not induce any changes in the number of apoptotic granulocytes, contrary to the earlier observations. The induction of programmed cell death in lymphocytes was also less intense after the infection with 72V/2003, since the three earlier investigated RHDVa caused more pronounced changes in the percentage of apoptotic lymphocytes during the entire time of the experiment (Niedźwiedzka-Rystwej *et al.,* 2013).

In summary, the results obtained in this study, in which the effect of seven haemaglutinating RHDV strains (including one antigenic variant) was investigated, confirmed that the infection with RHD virus induces apoptosis in peripheral blood granulocytes and lymphocytes. This effect is manifested by statistically significant increase in the percentage of apoptotic cells, observed as early as 4–8 h post infection, and maintained until 24–36 h after infection. However, the pattern of changes observed in case of infection with the antigenic variant 72V/2003 differs from the previously reported results regarding the infections with other haemaglutinating RHDVa (Vt97, Triptis, Hartmannsdorf). This demonstrates that the induction of apoptosis may be connected with the hemagglutination abilities of the RHD virus. Furthermore, it seems that the observed changes in the number of apoptotic cells in the course of RHDV infection, together with changes in the blood vessels resulting from DIC (Ueda *et al.,* 1992), and the quantitative and qualitative changes in the immunological activity of granulocytes and lymphocytes (Tokarz-Deptuła, 2009; Niedźwiedzka, 2010; Niedźwiedzka-Rystwej & Deptuła, 2010), are caused by the interaction of RHD virus with the host organism. The results clearly indicate that the ability of RHDV to induce programmed cell death in host granulocytes and lymphocytes has a direct influence on the pathogenesis of rabbit plague caused by this virus.

Results obtained in the presented study have shown that apoptosis is induced in the peripheral blood granulocytes and lymphocytes of rabbits infected with seven investigated haemaglutinating RHDV strains, originating from different regions of Europe (Hungary, Austria, France). This demonstrates that programmed cell death is involved in the viral infection, which is manifested by an increase in the percentage of apoptotic cells observed already between the 4th and 8th h post infection, depending on the RHD virus strain. The increase in the number of apoptotic cells is sustained until 24–36 h after infection, however, the process is more intense in the lymphocytes as compared to granulocytes, since more pronounced changes were noted throughout the experiment in the lymphocyte population. These results indicate a more important role of lymphocytes in the course of RHDV infection. Presented observations are partially in agreement with previous studies on the host immune responses to infections caused by RHDV antigenic variants, which also showed that biological properties of the virus determine the reaction of the immune system of the infected animals. It should be pointed out, however, that the haemagglutination abilities of the RHDV strains do not seem to play as important role in the regulation of the immune response as the biological properties of RHDVa (Tokarz-Deptuła, 2009; Niedźwiedzka-

Rystwej & Deptuła, 2010; 2012). Moreover, the studies by *Nyström et al.* (2011), confirm the hypothesis that the haemagglutination abilities of the virus depend more on the genotype of the specific RHDV strain than on any other feature of the RHDV strain (together with the immune response).

In conclusion, it was shown that changes in the number of apoptotic granulocytes and lymphocytes observed in rabbits experimentally infected with different RHDV strains used in this study, as well as in previous investigations (Table 1), play an important role in the pathogenesis of the rabbit plague. The process of programmed cell death, together with the pattern of changes in the populations of the immune cells in rabbits infected with different haemaglutinating and non-haemaglutinating RHDV strains (as well as antigenic variants) contribute to the mechanism of the pathogenic actions of the RHD virus.

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