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Original article

Apoptosis of granulocytes and lymphocytes in peripheral blood in rabbits infected with haemagglutinating and non-haemagglutinating antigenic variants of the RHD (rabbit haemorrhagic disease) virus

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Abstract

This paper attempts to study the dynamics of apoptosis of granulocytes and lymphocytes in peripheral blood in rabbits infected with haemagglutinating (Vt97, Triptis, Hartmannsdorf) and non-haemagglutinating (Pv97, 9905 RHDVa) antigenic variants of the RHD virus. The pathogenicity of those antigenic variants was also assessed by recording the mortality of the infected animals. The animals were infected with antigenic variants and blood was sampled at hour 0,4,8,12,24,36 p.i. and the percentage of apoptotic granulocytes and lymphocytes was measured with the use of flow cytometry. The results of the study showed that apoptosis is included during RHDV infection, as the number of apoptotic granulocytes and lymphocytes increases throughout the experiment; depending on the antigenic variant, apoptosis joins in at 4-8-12 h p.i. and lasts until 24-36 h p.i. Furthermore, the mortality of rabbits infected with the examined strains of RHD virus varied from 30% to 100%. This study performed for the first time in this manner, indicates the importance of apoptosis during infection with the RHD virus.

Key words: apoptosis, granulocytes, lymphocytes, RHD virus

Introduction

The RHD virus (rabbit haemorrhagic disease virus), belonging to the *Caliciviridae* family and causing plague among rabbits, is an interesting subject for study, due to its biological properties. It is differentiated in the aspect of pathogenicity (Chasey et al. 1995,

Capucci et al. 1998, Tokarz-Deptuła 1998, Tokarz-Deptuła et al. 2003, Hukowska-Szematowicz 2006, Matiz et al. 2006, McIntosh et al. 2007, Tokarz-Deptuła 2009), different haemagglutination capacity (Capucci et al. 1996, Kęsy et al. 1996, Schirmer et al. 1999, Prieto et al. 2000, Fitzner 2006, Tian et al. 2007), serological response (Berninger and

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Table 1. Characteristics of strains of the RHD virus, as used in the study.

No.	Name of the RHD virus strain	Biological property	Titre in the HA test	Country and year of origin of the RHD virus strain
1.	<i>Vt97</i>	Haemagglutinating antigenic variant	2560	Italy, 1997
2.	<i>Triptis</i>	Haemagglutinating antigenic variant	2560	Germany, 1996
3.	<i>Hartmannsdorf</i>	Haemagglutinating antigenic variant	2560	Germany, 1996
4.	<i>Pv97</i>	Non-haemagglutinating antigenic variant	negative	Italy, 1997
5.	<i>9905 RHDVa</i>	Non-haemagglutinating antigenic variant	negative	France, 1999

House 1995), and even antigenic variants have been selected within this virus (Capucci et al. 1998, Schirmer et al. 1999, Le Gall-Recule et al. 2003, Matiz et al. 2006, van de Bildt et al. 2006, Farnós et al. 2007, McIntosh et al. 2007, Tian et al. 2007). Recent immunological studies indicated that the RHD virus is “non-homogenous” and probably creates immunotypes (Tokarz-Deptuła 1998, Tokarz-Deptuła et al. 2002, Tokarz-Deptuła and Deptuła 2004, Hukowska-Szematowicz 2006, Tokarz-Deptuła 2009).

Studies comprising over 400 strains of the RHD virus described worldwide revealed that they have the capacity for clotting human red blood cells of 0 group at a temperature of 37°C, except for five strains of the virus – British Rainham (Capucci et al. 1996), Polish Blaszk (BLA) (Kęsy et al. 1996), Spanish Asturias (Prieto et al. 2000), German Frankfurt (Fra) (Schirmer et al. 1999), Chinese whn-1 (Tian et al. 2007), which do not have such a property, and two – Polish ŻD strain (Fitzner 2006) and German Hagenow (Schirmer et al. 1999) with intermediate features, which react variably in the haemagglutination (HA) test. This property of strains of the RHD virus, namely haemagglutination capacity, is related to the structure of protein VP60, in particular the location in this protein – in the P2 region at the N-terminal end of aminoacids of phenylalanine (304), alanine (305), serine (309), and at the C-terminal – of glycine (359), asparagine (365), alanine (365) and asparagine (386) (Tian et al. 2007). This property also conditions the “difference” of the strains of the RHD virus which were differentiated as antigenic variants (RHDVa), of which many have been described so far, including for example: Italian strains – Pavia97 (PV97) and Viterbo97 (Vt97) (Capucci et al. 1998), German strains – Triptis and Hartmannsdorf (Schirmer et al. 1999), French strains – 9905RHDVa, 00-Reu, 03-24, 01-38 (Le Gall-Reculé et al. 2003), Chinese strains – whn-1, whn-2, whn-3, YL, TP, WHNRH, CD, NJ1985, JXCHA97 (McIntosh et al. 2007, Tian et al. 2007), Hungarian strain – RH29/03 (Matiz et al. 2006), Cuban strain – CUB5-04 (Farnós et al. 2007), American strains – Iowa2000, IN05, NY01, UT01

(McIntosh et al. 2007), Dutch strains – NL2004-1, NL2004-2, NL2004-3 (van de Bildt et al. 2006), Polish strains – L145/04 and W147/05 and lately Korean strain – 06Q48-2, 08Q221, 08Q712, 08Q121, KV0801, 07Q92-1, 06D32-1, 06D106-1, 06Q755-1 (Oem et al. 2009).

It has been shown that strains of this virus cause various mortality, ranging from 70-100% (Tokarz-Deptuła et al. 2002, Tokarz-Deptuła and Deptuła 2004, Tokarz-Deptuła 2009), yet the highest pathogenicity was always shown by the strains appearing in the environment for the first time, although there have also been strains of the RHD virus the pathogenicity of which remains the same, regardless of the fact of their appearing in the environment. And so, for example, high mortality rate is caused by: haemagglutinating French Fr-1 strain – 90% mortality, Fr-2 – 100% (Tokarz-Deptuła 1998), Czech CAMP V-561 – 100% (Hukowska-Szematowicz 2006), Polish SGM – 95% (Tokarz-Deptuła 1998), Kr-1 – 95% (Tokarz-Deptuła et al. 2003), GSK – 93.8% (Tokarz-Deptuła 2009), ŻD – 100% (Tokarz-Deptuła 2009), as well as Italian strains of the RHD virus – 100% (Capucci et al. 1998), haemagglutinating Hungarian antigenic variants – 90% (Matiz et al. 2006), haemagglutinating British strains – 89-90% (Chasey et al. 1995) and rather haemagglutinating American strains – 70-95% (McIntosh et al. 2007), while the lowest mortality rate was recorded for haemagglutinating Polish PD strain – 25% (Tokarz-Deptuła 2009) of the RHD virus. Therefore, it can be assumed that within the RHD virus, there are more or less pathogenic strains, yet no-one has actually grouped them according to this property.

Studies on programmed death in rabbits infected with the RHD virus are few (Alonso et al. 1998, San-Miguel et al. 2006, Jung et al. 2000). Apoptosis in rabbits infected with the RHD virus – unspecified strains, was presented for the first time by Alonso et al. (Alonso et al. 1998), who, using histopathologic methods, pointed to characteristic degeneration of eosinophils and vacuolisation of hepatocyte cytoplasm, where aggregates of chromatin were detected,

significantly reduced nucleus and apoptotic bodies, as well as in macrophages in the lungs and kidneys, and blood monocytes. Other studies (Jung et al. 2000), with analogical methods, showed that the number of apoptotic hepatocytes increases in the course of infection of rabbits with the RHD virus, and their greater number was recorded at 30-31 h from infection and just before death. Also, the presence of typical apoptotic cells was detected in hepatocytes, macrophages of the abdominal cavity, epithelial cells, lungs, kidneys, heart, spleen, and lymph nodes (Jung et al. 2000). This phenomenon, based on activation of caspase 3 in hepatocytes, was studied by San-Miguel et al. (San-Miguel et al. 2006), who showed that the process of apoptosis after infection of rabbits infected with the Spanish AST/89 strain of the RHD virus begins at 36 and 48 h (San-Miguel et al. 2006).

In fact, apoptosis is listed as one of the elements of pathogenic action of viruses on the cells of the immune system, namely lymphocytes, and this has been described e.g. for the Ebola virus (Parrino et al. 2007). In the case of the latter virus, apoptosis in lymphocytes begins 5 days before death by decreasing the level of mRNA Bcl-2 expression (Parrino et al. 2007). Furthermore, in animals experimentally infected with the Ebola virus, it was shown that lymphocytes undergo apoptosis, which is also expressed in lymphocytopenia (Parrino et al. 2007). Such an observation regarding apoptosis of lymphocytes has been reported during human infection with HIV and SIV virus, as well as infection of pigs with African swine fever (Geisbert et al. 2000). According to data in the literature, apoptosis of immune system cells, particularly lymphocytes, can be induced by viral protein, chemical mediators freed from infected cells, and by interactions with other cells (Geisbert et al. 2000). Lymphocytes activated during viral infection can also enhance the induction of Fas and FasL, by which the apoptotic path is turned on (Geisbert et al. 2000). Hence, the quantitative record of this phenomenon in rabbits infected with the RHD virus, in the form of an increase in the volume of apoptotic granulocytes and lymphocytes, is the first, yet very important, step in considering the role of apoptosis in the mechanism of pathogenic impact of the RHD virus in the course of rabbit infection.

Previous studies on the RHD virus indicate that antigenic variants constitute a rather new problem, as not only are they interesting biological material, linking the properties of different antigenicity, and haemagglutination capacity, or the lack thereof, but the strains are also characterised with high malignity and seem to push out the "typical" strains from the natural environment. At the same time, the problem

of the apoptosis phenomenon, despite its unquestioned place in the pathogenic action of the virus, had not been studied before in reference to the cells of the immune system in the dynamic system. Therefore, the purpose of this study was to trace the dynamics of the phenomenon of apoptosis of granulocytes and lymphocytes in peripheral blood in rabbits infected with three haemagglutinating (Vt97, Triptis, Hartmannsdorf) and two non-haemagglutinating (Pv97, 9905 RHDVa) antigenic variants, for which additionally their pathogenicity was assessed by recording the mortality of the infected animals.

Materials and Methods

The study was performed on 100 mixed-breed rabbits of various sexes, weighing in the range 3.2-4.2 kg, marked as conventional animals, coming from a licensed breeding farm under continuous veterinary and zootechnical supervision (Niedźwiedzka-Rystwej and Deptuła 2010). During the experiment, the animals were housed at the vivarium of the Department of Microbiology and Department of Immunology, Faculty of Biology, University of Szczecin, where zootechnical parameters conformed to the appropriate standards in respect to: lighting, ventilation and size of cages (Niedźwiedzka-Rystwej and Deptuła 2010). After transport to the vivarium, the animals were subjected to a two-week adaptation period. The animals were fed with full-portion rabbit feed (16% Królik z Motycza) at 0.15-0.20 kg/day and had unlimited access to water. The animals were infected with RHDV strains defined as antigenic variants presented in Table 1. Each virus strain used in the study for experimental infection came from an animal that had died of natural causes in its country of origin. Livers obtained from these animals in the form of a homogenate, were used to infect laboratory rabbits, from which (after their death) the liver was sampled. The liver was then prepared as a 20% homogenate cleared by centrifugation at 3000 rpm, 10% chloroforming for 60 minutes and centrifugation again, and then suspension in glycerol in a 1:1 proportion (Fitzner et al. 1996). All the antigens prepared had the same number of viral particles specified with density 1.34 g/dm³.

The animals in infected groups (10 animals per each antigenic variant) were administered intramuscularly (muscles of the lower limb) a dose of antigen of the RHD virus suspended in 1 ml glycerol, while rabbits in control groups (also 10 animals per each antigen variant) analogically received 1 ml of glycerol. Blood for the tests was drawn both in infected and

control groups from the peripheral vein of the rabbit ear at hour "0", namely before administration of the RHD virus or glycerol, and at 4,8,12,24,36 h p.i. According to the recommendations of the Local Ethical Committee in Szczecin (permit no. 11/06), the experiment was terminated upon the occurrence of the first symptoms of the disease or in the event of animal death, which occurred at 24 or 36 h after administration of the RHD virus strain.

The phenomenon of apoptosis, using a set of ApoFluor®Green Caspase reagents (MP Biomedicals, USA) with a FACScan flow cytometer by Becton Dickinson, and Diva software, was assessed by recording the activity of the general pool of caspases 1,3,4,5,6,7,8 and 9 (MP Biomedicals, USA) in granulocytes and lymphocytes of rabbit peripheral blood. The result is shown as a percentage of apoptotic granulocytes and lymphocytes. Heparinised blood was centrifugated for 5 minutes at 400 xg, after which 300 µl of the suspension was transferred to Ependorf tubes and 10 µl of ApoFluor®Green was added, mixed and incubated for 60 minutes at a temperature of 37°C in the presence of 5% CO₂, mixing every 20 minutes. After this time, 2 ml of rinsing buffer was added to the mixture to remove colorant which was not bound to the cell, after which it was centrifuged for 5 minutes at 400 xg at room temperature. After discarding the supernatant, the washing was repeated and 400 µl of rinsing buffer and 2 µl of propidium iodide (PI) was added to the suspension. The prepared cell suspensions were incubated in ice for 10 minutes and analysed using a flow cytometer. The application of two colorants – ApoFluor®Green and PI allowed for differentiation between live (apoptotic) cells and dead (necrotic) cells, as the colorants stain in two different ranges of the spectrum – green and red. Interpretation of the results was performed by calculating, from the total pool of cells, the percentage of apoptotic granulocytes and lymphocytes, as the flow cytometer recorded both granulocytes and lymphocytes as Apo(+)/PI(-) cells – stained with ApoFluor®Green, while not stained with propidium iodide, and Apo(+)/PI (+) cells – stained both with ApoFluor®Green and PI, and these cells were treated as apoptotic. The results were subjected to statistical analysis with Student T-test at p=0.05 using Statistica software version 6.0, by comparing the results obtained in infected and control rabbits.

Results

The results obtained for apoptosis for the assessed antigenic variants (Triptis, Hartmannsdorf, Vt97, 9905 RHDVa, Pv97) (Fig. 1, 2) indicated that for all

the studied strains, apoptosis is expressed with an increase in the percentage of apoptotic granulocytes and lymphocytes from 4-8 h to 24-36 h p.i. And so, for haemagglutinating antigenic variant Triptis of the RHD virus, apoptosis of granulocytes begins from 8 h from antigen administration and lasts up to 36 h, while in lymphocytes, for this strain, apoptotic changes are recorded between 12 and 24 h p.i. In turn, for haemagglutinating antigenic variant Hartmannsdorf of the RHD virus, the percentage of apoptotic granulocytes increased at 12 and 24 h p.i., and only at 4 h p.i. in the case of lymphocytes. In the case of haemagglutinating antigenic variant Vt97 of the RHD virus, the changes in the percentage of apoptotic granulocytes occurred between 8 and 36 h p.i., and between 4 and 24 h p.i. for lymphocytes. For non-haemagglutinating antigenic variant 9905 RHDVa, both in the percentage of granulocytes and lymphocytes, apoptotic changes were recorded falling in the period from 8 to 36 h p.i. In turn, for the non-haemagglutinating Pv97 antigenic variant, an increase in apoptotic granulocytes occurred at 8, 12, 24, 36 h p.i., with an increase in apoptotic lymphocytes – at 24 and 36 h. To conclude, it must be noted that the frequency of apoptotic changes was greater in the case of granulocytes, while the number of cells on average remained at a level ten times lower as compared to apoptotic lymphocytes. Also, when analysing the percentage of apoptotic granulocytes, it was shown that the same number of changes, occurring at 8, 12, 24, 36 h from antigen administration, was recorded for haemagglutinating antigenic variants Vt97 and Triptis, and non-haemagglutinating Pv97 and 9905 RHDVa variants, while in the case of haemagglutinating antigenic variant Hartmannsdorf analogical changes occurred at 12 and 24 h from administration of the virus. In the case of the percentage of apoptotic lymphocytes, the greatest frequency was recorded for haemagglutinating antigenic variant Vt97 (4, 8, 12, 24 h) and non-haemagglutinating antigenic variant 9905 RHDVa (8, 12, 24, 36 h), while slightly less intensive changes were recorded for haemagglutinating antigenic variant Triptis (12, 24 h) and non-haemagglutinating antigenic variant Pv97 (24, 36 h), with the least intensive for haemagglutinating antigenic variant Hartmannsdorf (4 h).

As regards the mortality rate of rabbits infected with haemagglutinating and non-haemagglutinating antigenic variants, it must be stated that it varied, and amounted to 100% for haemagglutinating antigenic variants Triptis and Hartmannsdorf and non-haemagglutinating Pv97 variant, and 90% for non-haemagglutinating antigenic variant 9905 RHDVa, and 30% for haemagglutinating antigenic variant Vt97.

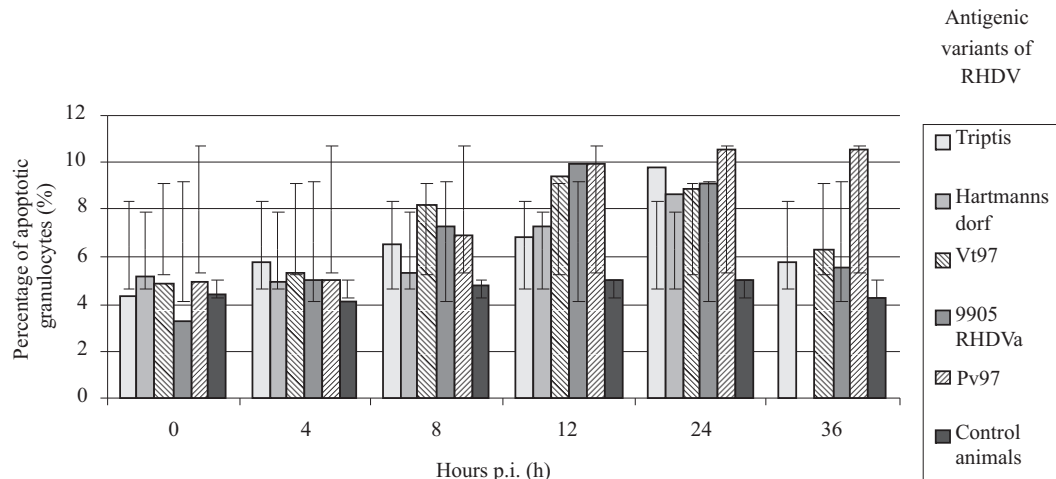


Fig. 1. Percentage of apoptotic granulocytes of peripheral blood in rabbits experimentally infected with antigenic variants of RHD virus.

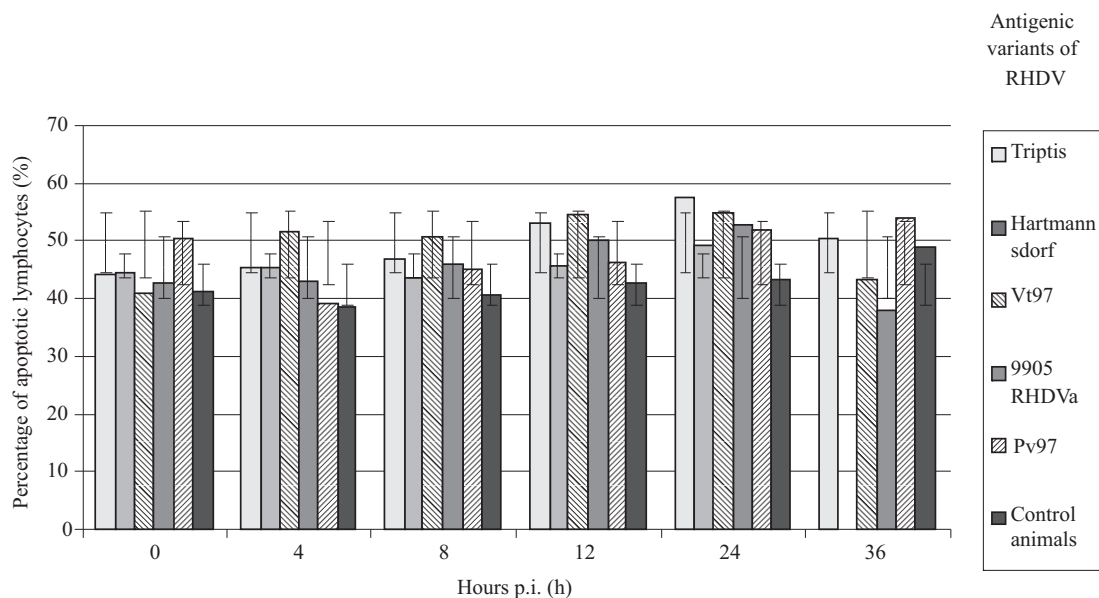


Fig. 2. Percentage of apoptotic lymphocytes of peripheral blood in rabbits experimentally infected with antigenic variants of RHD virus.

Discussion

A study on the phenomenon of apoptosis of granulocytes and lymphocytes in peripheral blood in rabbits infected with haemagglutinating (Triptis, Hartmannsdorf, Vt97) and non-haemagglutinating (9905 RHDVa, Pv97) antigenic variants, is presented for the first time in the literature. Although so far apoptosis had been assessed via histopathological studies (Alonso et al. 1998, Jung et al. 2000) and its inclusion in the “caspase” route was recorded in liver cells in rabbits infected with the Spanish AST/89 strain of the RHD virus (San-Miguel et al. 2006), such studies not only did not refer to haemagglutinating and non-haemagglutinating strains of the RHD virus, and the more

so to its antigenic variants, but, also, they were not performed in reference to the cells of the immune system in the dynamic system.

Results from this study indicate that apoptosis is included during infection with the RHD virus, as in the course of the infection the number of apoptotic granulocytes and lymphocytes increases. The results confirm to a certain extent previous studies (San-Miguel et al. 2006), but refer to the “typical” strain of the RHD virus, where participation of caspase-3 was noted at 36 and 48 h p.i. in rabbits experimentally infected with the Spanish AST/89 strain of the RHD virus. Therefore, it can be stated that infection of rabbits with the RHD virus causes such cells to be directed to the “caspase” route of programmed

death. Similar results were obtained in infection of rabbits with EBHS virus (European Brown Hare Syndrome) of the *Lagovirus* genus from the *Caliciviridae* family, where an increase in the percentage of apoptotic granulocytes was recorded at 12, 24 and 36 h, and lymphocytes from 8 to 60 h p.i. (Nowaczyk 2007). In relation to the above, it can be stated that the observed process of apoptosis in immune system cells in rabbits infected with haemagglutinating and non-haemagglutinating antigenic variants of the RHD virus is a new element of pathogenesis, which had not been given attention so far.

It must also be added that the property of haemagglutination capacity in the presently studied antigenic variants did not differentiate such strains, and also there is no interdependence between the intensity of apoptotic changes and the mortality rate of the infected rabbits, as only the haemagglutinating antigenic variant Vt97 caused lower mortality, while the remaining, both haemagglutinating and non-haemagglutinating antigenic variants caused 90-100% mortality of the infected animals. Such high mortality caused by most antigenic variants studied partly confirms the observations by other authors (Grazioli et al. 2000) reporting the possible replacement of classic strains of the RHD virus by antigenic variants.

The present study, performed for the first time, of the apoptosis of granulocytes and lymphocytes in the peripheral blood of rabbits infected with haemagglutinating and non-haemagglutinating antigenic variants, indicated that apoptosis is included during the infection with this virus depending on the antigen variant studied, at 4-8-12 h p.i., and lasts until 24-36 h, while in the course of the infection the number of apoptotic granulocytes and lymphocytes increases. At the same time, it must be stated that in the present study, it cannot clearly be assessed whether haemagglutination capacity has an impact on the method and time of inclusion of apoptosis in the course of infection with the RHD virus. Almost all the antigenic variants studied showed high a mortality rate, except for one haemagglutinating antigenic variant Vt97 (30%): this strain caused inclusion of apoptosis of cells of the immune system at a level not different from the remaining variants.

To conclude, the results of this study help to expand the knowledge on the pathogenic action of the RHD virus, and extend the knowledge of the strains of the RHD virus defined as antigenic variants, including both haemagglutinating and non-haemagglutinating strains.

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