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Indices of non-specific immunity: an element of natural immunity in rabbits infected with RHD (rabbit haemorrhagic disease) virus

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Abstract

The paper describes non-specific immunity mediated by parameters of cell and humoral immunity in rabbits experimentally infected with seven haemagglutinating strains of the rabbit haemorrhagic disease (RHD) virus, including one which is an antigenic variant of RHDV, originating from various European countries and with a different isolation time. In addition to the studies performed earlier on different strains of RHDV, this paper also shows that the immunological differences between the strains may be the core of the existence of immunotypes among RHDV.

Key words: *rabbit haemorrhagic disease virus, antigenic variant, immunotypes.*

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Introduction

The interest in the rabbit haemorrhagic disease (RHD) virus has not decreased since the end of the last century, when in 1984 the virus was identified in China [1–4]. Since then, the virus causing viral rabbit plague among both wild and farm animals has spread worldwide, posing a severe threat to the animals that have a number of applications in various countries – from reference laboratory animals to animals hunted for [1]. The studies on biology of the RHD virus are performed worldwide, although they are hampered significantly by the impossibility of its cultivation *in vitro* [1–4]. Immunological studies on the virus are even less popular. Apart from a few reports that have appeared immediately after registration of the virus in China [5–7] and papers by a Portuguese team [8, 9], only Deptuła's team [2–4, 10–23] deals with immunological differentiation among the strains of the RHD virus. These studies will lead to determining the existence of immunotypes among the virus, i.e. strains differing in immunological response, defined by parameters of non-specific and specific cellular and humoral immunity, currently referred to as natural and acquired immunity. In such studies [2–4, 10–23] regarding 24 strains of RHDV originating from various, mainly European countries, also differing as to biological property, namely haemagglutination capacity, differences in the immunological profile have been recorded. This differentiation, yet only regarding antigenic properties, is also confirmed by the studies pointing to the existence of antigenic variants (RHDVa) of the RHD virus [24].

The purpose of the study was to assess the parameters of non-specific immunity, both cellular and humoral, forming elements of natural immunity, that is polymorphonuclear leukocytes (PMN) adherence capacity, absorption index and percentage of absorbing cells, spontaneous, stimulated and spectrophotometric nitro blue tetrazolium (NBT) test, stimulation index, and spontaneous and stimulated metabolic activity coefficient of granulocytes (WAMG), as well as myeloperoxidase activity in PMN cells, and lysozyme concentration and activity in serum in rabbits infected with seven haemagglutinating strains of the RHD virus, including one that is an antigenic variant of RHDV (Table 1), originating from various European countries and differing in the year of isolation. Since now, the analysed strains have not been assessed in the immunological aspect,

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Table 1. Characteristics of rabbit haemorrhagic disease virus strains used for the study

No.	Name of the RHD virus strain	Biological property	Country and year of isolation of the RHD virus strain
1.	*01-04*	haemagglutinating	Austria, 2004
2.	*237/04*	haemagglutinating	Austria, 2004
3.	*V-412*	haemagglutinating	Austria, 1989
4.	*05-01*	haemagglutinating	France, 2005
5.	*24V/89*	haemagglutinating	Hungary, 1989
6.	*1447V/96*	haemagglutinating	Hungary, 1996
7.	*72V/2003*	haemagglutinating antigenic variant	Hungary, 2003

the results obtained will enrich the results regarding immunology of RHDV strains.

Material and methods

The study involved 70 mixed-breed rabbits weighing 3.2-4.2 kg, labelled as conventional animals, originating from a licensed farm, remaining under continuous veterinary and zoo-technical supervision [25]. During the experiment, the animals remained at the vivarium of the Department of Microbiology and Department of Immunology of the Biology Faculty at the University of Szczecin, where zoo-technical parameters were in line with the recommended Polish standards developed according to the European Union Directive as regards temperature and humidity, as well as lighting and size of cages for animals [26]. After transportation to the Department vivarium, the animals were given a two-week adaptation period. The animals were fed with all-mash rabbit feed (16% Królik z Motycza) of 0.15-0.20 kg/day, and had unlimited access to water. The rabbits were divided into groups of infected animals (5 animals each), and each group of infected animals had a corresponding group of control animals (5 animals each). The animals in infected groups were administered intramuscularly (lower limb muscles) a dose of antigen of RHDV suspended in 1 ml of glycerol, while rabbits in control groups received by analogy 1 ml of glycerol. Each of the viral strains (Table 1) originated from a naturally deceased animal. These strains, in the form of liver homogenate, were used for experimental infection of rabbits, from which liver was sampled after death. Next, it was prepared as 20% homogenate purified by centrifugation at 3000 rpm, 10% chloroforming for 60 minutes, and centrifugation again, and next by suspension in glycerol at the ratio of 1 : 1 [27]. All antigens prepared had the same number of viral particles determined with buoyant density in caesium chloride, within the limits of 1.34 g/dm^3 [27].

In blood, non-specific cellular and humoral immunity parameters were marked. And so, PMN cell adherence capacity was determined using the method of Lorente *et al.* [28], comprising calculation of the percentage of PMN cells adherence to glass balls, while PMN cells absorption capacity was assessed using the method developed by Brzuchowska and Ładosz, and modified by Deptuła [29], which involves calculation of absorption of reference *Staphylococcus aureus 209P* strain by PMN cells, and which was presented as an absorption index (IP), calculated as average volume of bacteria absorbed by one PMN cell in 100 consecutive cells, and the percentage of absorbing cells (% kp), calculated as the percentage of PMN cells that reveal absorption capacity per 100 consecutive PMN cells. Furthermore, in blood, capacity for nitro blue tetrazolium reduction (NBT) was assessed in PMN cells of peripheral blood using the cytochemical method in spontaneous and simulated test according to Park *et al.* [30] and using spectrophotometric method (according to Raman and Poland [31]). Moreover, metabolic activity coefficient of granulocytes (WAMG) was calculated according to Grządzielska [32], while the stimulation index (IS) – according to Lechowski [33].

As regards indices of non-specific humoral immunity, the assessment of myeloperoxidase (MPO) activity was assessed using the Graham method described by Zawistowski [34], which involves marking the activity of the enzyme in PMN cells by determination of colour intensity in the histochemical reaction, whereas MPO activity coefficient was expressed using a formula according to Afanasyev [35]. In turn, lysozyme (LZM) concentration in blood serum was determined using the clot diffusion method according to Hankiewicz [36], for reference the *Micrococcus lysodeikticus* strain, while lysozyme activity coefficient was calculated using the formula by Szmigielski [37]. All the results obtained were subject to a statistical analysis using *t*-Student test at $p = 0.05$ in the Statistica software v. 6.0, comparing the results obtained in infected and control rabbits.

Results

At first, it must be pointed out that the number of factors assessed in the area of natural immunity parameters (Table 2), namely indices of non-specific cellular immunity, was three times higher than indices of the immunity in the area of non-specific humoral immunity parameters, which resulted in more conclusions drawn on the basis of the first ones. And thus, as to results regarding non-specific cellular immunity parameters, in the case of all seven analysed strains (01-4, 237/04, V-412, 05-01, 24V/89, 1447V/96, 72V/2003) of RHDV, more statistically significant decreases than increases were recorded, while in the case of non-specific humoral immunity parameters, the image was the reverse, namely more statistically significant increases were recorded as compared to decreases.

Table 2. Results obtained in non-specific cell-mediated and humoral immunity in rabbits infected with 7 rabbit haemorrhagic disease virus strains

Strain **Parameters**	**Austrian 01-04**	**Austrian 237/04**	**Austrian V-412**	**French 05-01**	**Hungarian 24V/89**	**Hungarian 1447V/96**	**Hungarian 72V/2003**
PMN cells adherence capacity	↑ – ↓ 24	↑ – ↓ 12, 24	↑ 24 ↓–	↑ – ↓ 8, 24, 36	↑ 8, 12, 24 ↓ –	↑ 12 ↓ –	↑ – ↓ –
Absorption index	↑ – ↓ –	↑ 8 ↓ –	↑ – ↓ 8, 12, 24	↑ – ↓ –	↑ – ↓ 12, 24	↑ – ↓ –	↑ – ↓ 24
Percentage of absorbing cells	↑ – ↓ -	↑ – ↓ -	↑ – ↓ 24	↑ – ↓ 12, 24, 36	↑ – ↓ 8, 12, 24	↑ – ↓24	↑ – ↓ 24
NBT spontaneous test	↑ – ↓ 12, 24	↑ – ↓ 8, 12, 24	↑ 24 ↓ –	↑ 24, 36 ↓ –	↑ 12, 24 ↓ –	↑ – ↓ 24	↑ – ↓ 24
NBT stimulated test	↑ – ↓ 8,12	↑ 12 ↓ 24	↑ – ↓ –	↑ 24 ↓ –	↑ – ↓ –	↑ – ↓ 24	↑ – ↓ 24
NBT spectro-photometric test	↑ 24 ↓ –	↑ 12, 24 ↓ –	↑ 24 ↓ –	↑ 36 ↓ –	↑ – ↓ 8	↑– ↓ –	↑ – ↓ –
Stimulation index	↑ 12, 24 ↓ –	↑ 8, 12, 24 ↓ –	↑ – ↓ –	↑ – ↓ –	↑ – ↓ –	↑ – ↓ –	↑ – ↓ –
Spontaneous metabolic activity coefficient of granulocytes	↑ – ↓ –	↑ – ↓ 12, 24	↑ – ↓ –	↑ 24 ↓ –	↑ 8, 24 ↓ –	↑ 12 ↓ 24	↑ – ↓ 24
Stimulated metabolic activity coefficient of granulocytes	↑ – ↓ 8, 12	↑ – ↓ –	↑ – ↓ –	↑ 24 ↓ 8	↑ 12 ↓ –	↑ 12 ↓ 24	↑ – v 24
Myeloperoxidase activity	↑ – ↓ –	↑ 8 ↓ –	↑ 8 ↓ –	↑ 24, 36 ↓ –	↑ 8, 12 ↓ –	↑ 8 ↓ 24	↑ – ↓ 12, 24
Lysozyme concentration	↑ 24 ↓ –	↑ – ↓ 8, 12	↑ – ↓ –	↑ 12, 24 ↓ –	↑ – ↓ –	↑ – ↓ –	↑ 24 ↓ –
Lysozyme activity	↑ 24 ↓ –	↑ – ↓ –	↑ 12, 24 ↓ –	↑ 12, 24 ↓ –	↑ – ↓ –	↑ – ↓ 8, 12	↑ 24 ↓ –

Legend: Arrows represent statistically significant increase (↑) or decrease (↓) of the parameter. Numbers standing by the arrows represent the hour of testing (0, 4, 8, 12, 24, 36 h)

In the case of haemagglutinating Austrian strain 01-4 of RHDV, increases were observed for spectrophotometric NBT test (24 h) and stimulation index (12, 24 h), while statistically significant decreases were recorded for PMN cell adherence capacity (24 h), spontaneous NBT test (12, 24 h), and stimulated metabolic activity coefficient of granulocytes (WAMG) (8, 12 h). In turn, no changes were manifested by this strain in such factors as absorption index and absorption cell percentage, as well as spontaneous WAMG parameter. As to analysed parameters describing non-specific humoral immunity in the case of strain 01-4 of RHDV, increases at 24 h were observed for lysozyme concentration and activity, while MPO coefficient remained unchanged.

As to changes recorded for parameters of non-specific cellular immunity in the case of haemagglutinating Austrian strain 237/04 of RHDV, statistically significant increases were recorded for absorption index (8 h), stimulated NBT test (12 h), stimulation index (8, 12, 24 h), while decrease was observed for such parameters as adherence capacity (12, 24 h), spontaneous NBT test (8, 12, 24 h), stimulated NBT test (24 h), and spontaneous WAMG (12, 24 h). In the case of this strain, the percentage of absorbing cells and stimulated WAMG remained unchanged. In turn, in the case of non-specific humoral immunity parameters, the strain caused an increase in MPO activity at 8 h, decrease in lysozyme concentration at 8 and 12 h and no changes to lysozyme activity.

As regards Austrian haemagglutinating strain V-412 of RHDV, it must be stated that the strain caused an increase falling exclusively at 24 h from infection in the case of parameters such as adherence capacity, spontaneous and spectrophotometric NBT test, while decrease was recorded for absorption index (8, 12, 24 h) and the percentage of absorbing cells (24 h). As regards stimulated NBT test, stimulation index, as well as spontaneous and stimulated

WAMG in the case of this strain of RHDV, no statistically significant changes were recorded. In turn, in the case of non-specific humoral immunity parameters, the strain caused an increase in MPO activity at 8 h, decrease in lysozyme activity at 12, 24 h and no changes to concentration of the latter enzyme.

In the case of changes recorded for French haemagglutinating strain 05-01 of RHDV for non-specific cellular immunity parameters, increases were observed in spontaneous NBT test (24, 36 h), stimulated NBT (24 h), spectrophotometric NBT (36 h), spontaneous and stimulated WAMG (24 h), while decreases were obtained for such parameters as adherence capacity (8, 24, 36 h), absorbing cell percentage (12, 24, 36 h), and stimulated WAMG (8 h). In this case of RHDV, no changes were recorded for the absorption index and stimulation index. In the case of changes to non-specific humoral immunity parameters with reference to strain 05-01 of RHDV, only increases were observed, and so was in the case of MPO activity coefficient at 24, 36 h, while in the aspect of lysozyme concentration and activity at 12 and 24 h.

As regards changes caused by Hungarian haemagglutinating 24V/89 strain of RHDV for non-specific cellular immunity parameters, statistically significant increases were recorded for adherence capacity (8, 12, 24 h), spontaneous NBT test (12, 24 h), spontaneous WAMG (8, 24 h) and stimulated WAMG (12 h). In turn, decreases fell at 12, 24 h for the absorption index, 8, 12, 24 h for absorbing cell percentage, and 8 h for spectrophotometric NBT test. No changes were recorded for stimulated NBT test and stimulation index. In turn, in the case of non-specific humoral immunity parameters of strain 24V/89, increase in MPO activity was observed at 8, 12 h, and no changes for lysozyme concentration and activity.

Analysing the changes in non-specific cellular immunity parameters for Hungarian haemagglutinating 1447V/96 strain of RHDV, it must be stated that the strain caused an increase falling exclusively at 12 h for adherence capacity, spontaneous and stimulated WAMG, while decreases were only recorded at 24 h for absorbing cell percentage, NBT test, spontaneous and stimulated WAMG, while no changes were revealed for the absorption index, spectrophotometric NBT test, and stimulation index. In turn, as regards non-specific humoral immunity parameters, 1447V/96 strain showed increases in the MPO activity at 8 h, and decreases for the same parameter at 24 h, and lysozyme activity at 8, 12 h, as well as no changes to lysozyme concentration.

The last of the currently analysed strains of RHDV was Hungarian haemagglutinating antigenic variant 72V/2003, which within the parameters of non-specific cellular immunity caused a special picture as changes were only recorded in the form of decrease falling exclusively at 24 h for parameters such as absorption index and absorbing cell percentage, NBT test, and spontaneous and stimulated WAMG. In turn, in the case of other parameters (adherence capacity, spectrophotometric NBT test, stimulation index), no changes were recorded. In turn, in the case of non-specific humoral immunity parameters, antigenic variant 72V/2003 caused an increase in lysozyme concentration and activity falling at 24 h, as well as decrease to MPO activity recorded at 12 and 24 h.

Discussion

When analysing the obtained results, it must be stated that the currently performed experiment referring to the same immunological parameters as those evaluated in previous studies by our team [2-4, 10-23], when analysing immunogenicity of various strains of RHDV. The present study was to complete the immunological image of RHDV strains with new data, concerning new, non-analysed in this aspect, 7 strains of RHDV. It must be stated that the results of immunological studies regarding 24 strains of RHDV, including two haemagglutinating French strains (Fr-1, Fr-2) [2, 4, 18, 20] and one non-haemagglutinating antigenic variant 9905 RHDVa) [16, 27], seven haemagglutinating Polish strains (SGM, MAŁ, KGM, ŻD, PD, GSK, Kr-1) and one non-haemagglutinating one (BLA) [2, 4, 14, 19, 22, 23], four haemagglutinating Czech strains (CAMP V-351, CAMP V-561, CAMP V-562, CAMP V-558) [12, 13, 23], three Italian strains (haemagglutinating BS89 and antigenic variants – haemagglutinating Vt97 and non-haemagglutinating Pv97), one English non-haemagglutinating Rainham strain, four German strains (Hagenow with variable haemagglutination capacity, haemagglutinating antigenic variants Triptis and Hartmannsdorf, and non-haemagglutinating Frankfurt), and one Spanish non-haemagglutinating Asturias strain [17, 21, 22, 27], confirm that the diverse immunogenicity among such RHDV strains not only depends on the year and country of isolation, but also on biological properties, namely haemagglutination capacity, or formation of antigenic variants. This proves that the results can provide the basis for differentiation of immunotypes among such RHDV strains. In turn, when analysing presently obtained results for seven strains, it must be stated that in natural immunity factors, determined using the parameters of non-specific cellular immunity, principally a decrease in the parameters was recorded, while in the parameters of non-specific humoral immunity – their increase. Such picture indicates that during the infection with these strains of RHDV, activity of the immune system cells is weakened due to the activity of the virus, and at the same time, the presence of the virus in the organism causes secretion of antimicrobial substances recorded in the volume and activity of lysozyme, and MPO activity – indices of non-specific humoral immunity. Moreover, it was determined that most changes, both regarding natural immunity factors measured using the parameters of non-specific cellular and humoral immunity, regardless of the seven analysed RHDV strains, were recorded at 24 h from rabbit infection with such strains. Such a condition may indicate that the end of the first day of infection is the culmina-

tion moment for the appearance of visible changes to the immune system of rabbits due to virus activity. It must also be stated that the results in the natural immunity, but measured using the parameters of non-specific cellular immunity, allowed for grouping the analysed RHDV strains depending on the number of changes caused into two groups: strains causing more changes (Austrian haemagglutinating 237/04, Hungarian haemagglutinating 24V/89, and French haemagglutinating 05-01), and strains causing fewer changes (Austrian haemagglutinating 01-04, Hungarian haemagglutinating 1447V/96, Austrian haemagglutinating V-412, as well as Hungarian haemagglutinating antigenic variant 72V/2003). In turn, as to the immunity measured using the parameters of non-specific humoral immunity, differentiation was also recorded among the strains analysed, whereas most changes were recorded for haemagglutinating French strain 05-01, while the remaining six strains (Austrian haemagglutinating 01-04, 237/04, V-12, Hungarian haemagglutinating 24V/89, 1447V/96, and Hungarian haemagglutinating antigenic variant 72V/2003) formed a group of strains causing fewer changes, which confirms previous observations of our team [2-4, 10-23] evidencing the presence of immunotypes within RHDV. It must also be stated that in the present studies, no impact was recorded of such properties as the time and country of isolation of a particular RHDV strain on their immunogenicity, namely formation of immunogroups, similarly to haemagglutination capacity of RHDV, although previous studies [2, 27] show that the latter property can be important for immunotype formation within RHDV. Furthermore, the results of the present study indicate that the only analysed antigenic variant 72V/2003 of RHDV caused a different image of the analysed indices as compared to the remaining six currently analysed strains of RHDV, as only decreases in the parameters analysed were observed, which confirms prior studies by our team [2-4, 10-23] on the biology of antigenic variants of RHDV. Therefore, it can be concluded that the different response of the immune system should constitute a characteristic feature of antigenic variants of RHDV, apart from the specific "nature" of binding to antibodies, belonging to one genogroup and causing high mortality, which differentiates RHDVa from classic strains of RHDV.

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The authors declare no conflict of interest.

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