

CLINICAL CHEMISTRY, HAEMATOLOGY, IMMUNE RESPONSE AND HISTOLOGICAL EVALUATION OF RABBITS AFTER IMMUNISATION AND CHALLENGE WITH RABBIT HAEMORRHAGIC DISEASE (RHD) VIRUS

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Abstract: Following their immunisation and infection with a VSHI-CN-6 viral strain, a group of 15 rabbits were examined in a study of Rabbit Haemorrhagic Disease (RHD). Serum samples were collected from the external ear vein at 0, 15, 30 and 60 days post-immunisation. The recorded platelet numbers were closer to the lower physiological limit, indicating a mild thrombocytopenia, with values ranging from 26.6 to 30.43×10⁴/mm³. The phagocytic index revealed significant differences ($P<0.001$) between the mean values obtained before vaccination (day 0) and the 3 post-vaccination measurements, confirming the increase in phagocytic capacity after immunisation. Additionally, the serum lysozyme average value equalled 9.14 mg/mL post-vaccination. The analysis of variance revealed significant statistical differences ($P<0.05$) between the average values obtained before vaccination (0) and the post-vaccination values, measured on day 14 and 30, respectively. The morphology of the samples collected from the main organs involved in immune protection, spleen and gastric and portal lymph nodes highlighted changes corresponding to the post-vaccination immunological response. The white pulp of the spleen appeared as a diffuse lymphoid tissue, presenting with primary and secondary lymphoid follicles. The ratio of white/red pulp was in favour of the white pulp and multiple lymphoid follicles were present, indicating their reactivation. In the medullary area of gastric and portal lymph nodes, narrow lymphoid cords, circumscribed by relatively large lymphatic sinuses, and well defined lymphocytolysis were observed. Moreover, the exudate and lymphoid follicles during activation were noted in the cortical area. Furthermore, the inflammatory processes were identified, morphologically manifested by the thickening of connective tissue in the lymph node capsule, dilacerations of the connective fibres and the presence of light acidophilic serous exudate with rare inflammatory cells (serous lymphoreticulitis).

Key Words: rabbit haemorrhagic disease (RHD), clinical chemistry, haematology, immune response, histology, rabbits.

INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly infectious and fatal (80-100%) rabbit disease reported for the first time in Asia, which subsequently advanced in Australia and then in Europe (Liu *et al.*, 1984; Morise *et al.*, 1991; Villafuerte *et al.*, 1994, 1995; Strive *et al.*, 2009; Carvalho *et al.*, 2017). Infected rabbits usually die within 48-72 h,

and the disease is still responsible for significant economic losses and heavy mortality among wild and domestic rabbit populations (Chasey, 1997; Marchandeanu *et al.*, 1998; Mutze *et al.*, 1998).

The aetiological agent, Rabbit Haemorrhagic Disease Virus (RHDV), belongs to the genus *Lagovirus*, within the *Caliciviridae* family (OIE, 2016). This genus comprises several non-pathogenic rabbit caliciviruses, which are genetically related to, but relatively distant from RHDV, and the European Brown Hare Syndrome Virus (EBHSV) (Ohlinger *et al.*, 1990; Ohlinger and Thiel, 1993).

Structurally, RHDV is a non-enveloped icosahedral single-stranded positive-sense RNA virus. The virus capsid is about 40 nm in diameter and includes 90 dimers of a single capsid subunit, namely the VP60 protein. Like most caliciviruses, RHDV cannot be grown in cell culture. This feature severely inhibits the ability to study these viruses, as well as the development of specific control measures (Abrantes *et al.*, 2012). A major advance in the field was the finding that expression of recombinant VP60 protein in insect cells resulted in the formation of Virus-Like Particles (VLPs) that are morphologically and antigenically identical to the infectious RHDV virions (Parra and Prieto, 1990).

In recent decades, the RHDV capsid gene has been successfully used and described in many other heterologous systems (Barcena *et al.*, 2004). Until now, the VP60 protein has been derived from diverse sources, such as recombinant virus based systems (Sibilia *et al.*, 1995), which included baculoviruses (Nagesha *et al.*, 1995), *Escherichia coli* (Bertagnoli *et al.*, 1996), *Saccharomyces cerevisiae* (Boga *et al.*, 1997) and pox viruses (Fischer *et al.*, 1997), and even from plant sources (Castañón *et al.*, 1999).

Recombinant VP60 virus obtained in all these systems has been shown to provide immunity against lethal RHDV in rabbits. Both the virus-like particles and the recombinant vaccine virus VP60 have been shown to induce the establishment of protection against RHDV when given orally (Plana-Duran *et al.*, 1996).

It has been found that RHDV VLPs induced full immunity in rabbits against the destructive action of RHDV. These VLPs have also been used for the development of sensitive and reliable tests for detection of antibodies against RHDV (Robinson *et al.*, 2002). As *in vitro* systems are not suitable for efficient spreading of the virus, commercial vaccines against RHD were created from the liver of experimentally infected rabbits (Argüello Villares, 1991). Subsequently, inactivated vaccines against RHDV were introduced in the early 1990s and provided general cover, as all circulating strains were classified within a single serotype. These vaccines aided the control of RHD in rabbit farms, but studies in this domain are still not completed. It has been confirmed that ELISA methods developed for the diagnosis of RHD in domestic rabbits constitute applicable tools for RHDV monitoring (Cooke *et al.*, 2000; Robinson *et al.*, 2002).

In our study, we investigated changes in clinical chemistry, haematology, antibody levels and morphology of the restructuring lymphoid organs after immunisation against RHD and experimental infection with RHDV.

MATERIALS AND METHODS

Prior to the start of experiments, rabbits were kept for one week in the animal facility to allow adequate adaptation. All procedures were carried out in accordance with Directive 2010/63/EU on the handling of animals used for scientific purposes, the NRC guide and Romanian Law n° 2001/471 on Protection of Animals Used for Scientific or Other Experimental Purposes (Directive 2010/63/EU; NRC, 1996; Romanian Government, 2002). The study was approved by the Bioethics Commission of the Faculty of Veterinary Medicine, Timișoara.

Animals

In this study, 15 healthy rabbits (*Oryctolagus cuniculus*), 6-mo old Dwarf Hotot males, weighing about 1.000 ± 0.065 kg, provided by an authorised commercial breeder from Timiș County, Romania, were used. The observation time was 60 d post-immunisation; the animals were kept in restricted environment in the laboratory animal facility at the Faculty of Veterinary Medicine, Timișoara. Rabbits were housed in galvanised wire cages with compact ruptured floor surface, 3 rabbits / cage (762×762×460 mm) (l×w×h). During the experiment, the light cycle was 17/7 h light/dark cycle (to 30 lux ambient light), with $16 \pm 2^\circ\text{C}$ controlled environmental temperature and with relative humidity of $55 \pm 10\%$. As bedding, sterilised wood shavings (not pinewood) were used and changed twice a week. Granulated forage, balanced for vitamins, micronutrients and amino acids, was given *ad libitum* with NC4220 (Agroland, Romania) standard diet

for this rabbit category and water was provided via a nipple drinking system to each cage. The animals were kept in a quiet environment.

Immunisation and infection

The vaccine against rhdv was provided by Romvac Company Romania under the trade name Hemovirovac and administered at 0.5 mL dose/rabbit, subcutaneous, at day 0 and day 28 of the experiment. The inactivated vaccine contains the strain VSHI-CN-6, with a titre before inactivation of at least 1024 haemagglutination units (HAU). The rabbits were intranasally infected after 10 d of immunisation by administering a single dose of 0.5 mL liver homogenate provided by the Pasteur Institute of Bucharest. Blood samples were taken from the external ear vein at days 0, 15, 30, and 60 post immunisation (dpi) to evaluate the immune response against RHDV.

Laboratory methodology

The complete blood count (CBC) was determined using an automated haematology analyser MS 9-VET (MS Laboratories).

The serum properdin was colorimetrically determined, through isolation and complexing upon inulin, followed by treatment with Biuret reagent. Values were interpreted comparatively to a previously established standard curve.

Serum lysozyme was determined by the simple radial diffusion test in agar gel, using a culture of *Micrococcus lysodeicticus*. The diameter of the radius created by the lysis due to the microorganisms included in the medium was proportional to the lysozyme's concentration in the serum sample. Values were interpreted comparatively to a standard curve.

Phagocytic index was measured versus a reference strain of *Staphylococcus* sp. When evaluating results, the number of colony forming units (CFU) of the initial suspension of *Staphylococcus* sp., was compared to the CFU number from the whole blood mixture in a *ratio* of 1:1 after a 60 min incubation at 37°C.

The Phagocytic *ratio* and the Phagocytic *index*, were calculated after the known formulas (Equation 1 and Equation 2) and finally the obtained values were statistically analysed.

$$\text{Phagocytic ratio} = \frac{\text{The number of phagocytic cels overthrown bacteria}}{\text{Number of phagocytes}} \quad (1)$$

$$\text{Phagocytic index} = \frac{\text{The number of overthrown bacteria}}{\text{phagocytic cell}} \quad (2)$$

Antibody titre was determined by sandwich ELISA and the levels were expressed in OD (optical density) rates. The principle of the method consists of determination of specific antibodies using enzyme-labelled species anti-immunoglobulins. The antigen was obtained by homogenisation of the organs (liver), clarification, and dilution in phosphate buffered solution (PBS, Sigma Aldrich) containing 0.1% Tween 20.

Haemagglutination (HA) antibodies were determined by haemagglutination reaction. The viral Haemagglutination Inhibition Assay (HAI) was based on the ability of antibodies from the serum to inhibit the haemagglutinating action of virus. The HAI was performed using an antigen prepared by the Pasteur Institute, Bucharest. Serial dilutions of virus were prepared in U- or V-bottom shaped 96-well microtitre plates. The most concentrated sample in the first well was diluted to 1/5× of the stock, and then in the subsequent wells, twofold dilutions were used (1/10, 1/20, 1/40, etc.). The well with no virus added served as a negative control. Following serial dilutions, a standardised concentration of RBCs was added to each well and mixed gently and the plate was incubated for 30 min at room temperature. Following the incubation period, the agglutinated and non-agglutinated wells were distinguished. Titration of the antigen was performed according to the method described above; the titre was given by the last dilution of the virus that caused haemagglutination. Prior to testing, serum samples were inactivated by incubation for 30 min at room temperature, and inactivated samples were adsorbed with human serum blood group as follows: 100 µL of serum+100 µL red blood cells from suspension used for HA. Finally, serum was stored overnight at 40°C, during which red blood cells precipitated and the serum remained clear and was used in this form in the HAI (no centrifugation needed).

Blood and organs collection

To assess the post-vaccination reaction, we collected samples of blood (Brown, 2016; van Praag, 2016) and spleen and gastric and portal lymph nodes for histologic examination. To this end, 5 rabbits, one from each cage, were randomly chosen and euthanised using the intraperitoneal method, Euthazol vet (Produlab Pharma B.V., The Netherlands) in a dose of 100 mg/kg body weight (AVMA, 2013; University of Minnesota, 2014).

Histological examination

For histopathological examination, the spleen and gastric and portal lymph nodes were collected. They were collected at day 60 after vaccination, fixed (AFA - alcohol, formalin, and acetic acid) and then embedded in paraffin. Paraffin blocks containing tissue fragments were sectioned by microtome, resulting in 5 µm thick sections, mounted on glass slides with Mayer's albumin and stained by the Haematoxylin – Eosin - Methylene blue, (H&E-methylene blue) trichrome staining. Here the nuclei are violet, cytoplasm is blue-grey and collagen fibres are blue. Sections were examined under microscope according to the tissue type, with objectives ×20 and ×40, and the images were processed with a CX41 microscope (Olympus), equipped with image capture and data interpretation software.

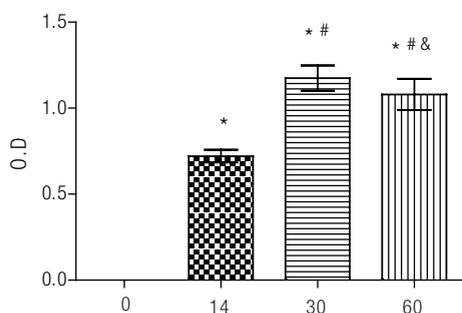


Figure 1: Statistical comparative values of the antibody titre expressed in optical density (OD) determined by sandwich ELISA. *Comparative with day 0; $P < 0.05$. #Comparative with day 14; $P < 0.05$. &Comparative with day 30; $P < 0.05$. Bars represent standard error of the mean.

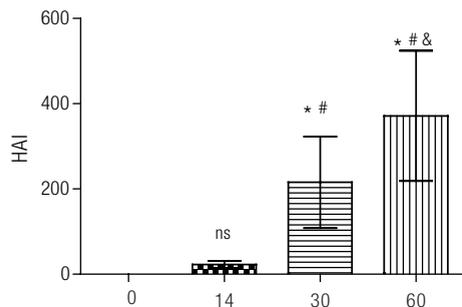


Figure 2: Statistical comparative values of the Haemagglutination Inhibition Assay (HAI). nsComparative with day 0; $P > 0.05$. *Comparative with day 0; $P < 0.05$. #Comparative with day 14; $P < 0.05$. &Comparative with day 30; $P < 0.05$. Bars represent standard error of the mean.

Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM). Graph Pad Prism (version 6.0 - GraphPad Software, San Diego, CA, USA) was used to compare results, using one-way analysis of variance (ANOVA) and Bonferroni's relation it was used as the *post hoc* test. Probability values of less than $P < 0.05$ were considered significantly different.

RESULTS AND DISCUSSIONS

Clinical observations

The animals tolerated well the small amounts of vaccine administered. No swelling or other general or local reactions were observed in this study. After the experimental infection, no animal showed clinical signs, and in only a few transient cases a mild epiphora, rhinorrhoea and sub-febrile status were recorded.

Serology

The antibody titre values were high, showing significant increases during the experimental periods. The antibody OD values and results for HAI were noted and statistically analysed. The results are presented in Figures 1 and 2.

Haematological changes, serum lysozyme and properdin

The results obtained on the evolution of blood and haemato-immunological parameters showed changes during the experimental period. Paraclinical significant differences from one parameter to another are presented in Table 1.

Table 1: Blood count, serum lysozyme and properdin values observed in 15 rabbits at days 0, 14, 30 and 60 post immunisation.

Specification/day	0	14	30	60	Specification/day	0	14	30	60
Haematocrit (HT) (%)					Thrombocytes (PLT) ($\times 10^4/\text{mm}^3$)				
Mean	36.91 ^a	39.14 ^b	40.30 ^c	40.82 ^d	Mean	26.63 ^a	29.96 ^b	30.43 ^b	30.18 ^b
SD	0.45	0.32	0.45	0.52	SD	0.49	0.80	0.80	0.77
Haemoglobin (Hb) (g/dL)					Mean platelet volume (MPV) (μm^3)				
Mean	9.27 ^a	10.35 ^b	10.62 ^b	10.58 ^b	Mean	3.09 ^a	4.03 ^b	4.04 ^b	4.13 ^b
SD	0.08	0.45	0.61	0.60	SD	0.24	0.22	0.16	0.25
Erythrocytes (RBC) ($\times 10^4/\text{mm}^3$)					Serum lysozyme ($\mu\text{g}/\text{mL}$)				
Mean	5.72 ^a	5.99 ^b	5.95 ^b	5.94 ^b	Mean	9.14 ^a	11.88 ^b	13.90 ^c	9.64 ^a
SD	0.31	0.15	0.13	0.15	SD	0.89	1.10	0.89	1.76
Mean corpuscular haemoglobin (MCH) (g/dL)					Serum properdin (mg/mL)				
Mean	26.25 ^a	27.76 ^b	27.75 ^b	27.99 ^b	Mean	19.23 ^a	21.34 ^b	23.85 ^c	21.37 ^d
SD	0.34	0.58	0.61	0.55	SD	1.38	0.99	0.59	1.41

Means followed by different superscript letters in the same row differ significantly to $P < 0.05$ by ANOVA test; SD: standard deviation.

Haematocrit and Haemoglobin

We believe that the statistical differences ascertained during the experimental period were subsequent to the IgM and IgG reaction pending the virus making its way into the cell, when the immune response to the protein fraction in pericapsid enclosed in the viral vaccine turned into cellular.

Although the haematocrit values were mildly increased during the experimental period, they were within the physiological limits for the species. Values obtained after vaccine administration corresponded to the previously published data, where in similar conditions the haematocrit ranged between 36.0 and 41.6% or 39.0 to 55.0% in rabbits 3-6 mo old. (Giammarco *et al.*, 2012; Laboklin, 2009).

Erythrocytes

The differences between values we obtained post-vaccination for erythrocytes were not significant statistically.

Platelets (thrombocytes)

The values noted were closer to the lower physiological limit, indicating a mild thrombocytopenia. Our values, ranging from 26.6 to 30.43 $\times 10^4/\text{mm}^3$, corresponded to the wider margin of values reported by other scientists, ranging from 12.0 to 50.0 $\times 10^4/\text{mm}^3$ (Chineke *et al.*, 2006; Părvu *et al.*, 1984).

Mean corpuscular haemoglobin, Mean platelet volume

Registered values lower than those reported in the literature (Chineke *et al.*, 2006; Părvu *et al.*, 1984, Özkan *et al.*, 2012). Statistical differences were ascertained between time 0 and other times post-vaccination.

Phagocytic index

A main indicator of phagocytic capacity of the specialised cells, amongst which the most important are the polymorphonuclear cells (e.g. macrophages, heterophiles and monocytes). Following the data analysis, the phagocytic index did not reveal significant differences between the four experimental periods, but highly significant statistical differences ($P < 0.001$) were observed between the mean values obtained before

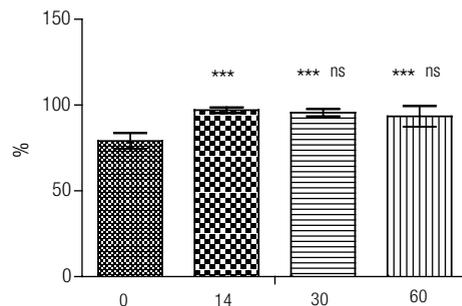


Figure 3: Statistical comparative values of the phagocytic index. ***comparative with day 0; $P < 0.001$. ^{ns}comparative with day 14 and 30. Bars represent standard error of the mean.

vaccination (day 0) and the 3 post-vaccination measurements, proving the increase of the phagocytic capacity after immunisation

The increase in the phagocytic index values, registered after the primary antigenic stimulus, indicated an intense level of antigen reception and processing. This property was maintained throughout the experimental period.

Lysozyme

A parameter used for assessing both the adaptive and natural immune responses induced by the immunogenic structures. In the data presented, initially, an average value of 9.14 mg/mL of serum lysozyme was registered. In this case, the analysis of variance did not reveal statistical differences ($P>0.05$) in the studied group between the serum lysozyme values from before the vaccination and the 3 post-vaccination periods. However, post-vaccination significant differences ($P<0.05$) were present between the average values obtained before vaccination (0) and the 2 post-vaccination periods, day 14 and 30 respectively.

Properdin

As a humoral factor involved in the cellular activation processes, properdin is considered an immunological reactivity indicator. The total properdin and properdin titre increased after the antigenic stimulus, having an important role in the activation of C_3 complement component. We assumed that the mean properdin values decreased at the end of the

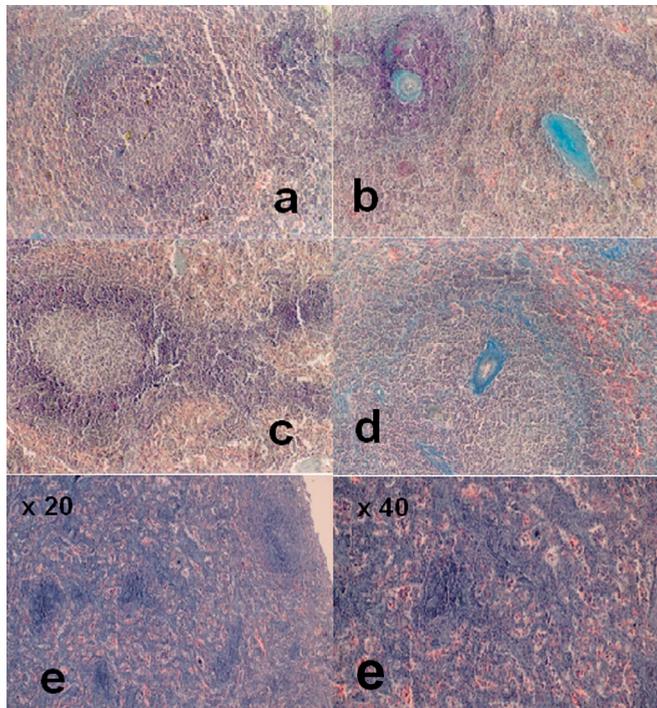


Figure 4: Rabbit spleen Haematoxylin – Eosin - Methylene blue (H&E-methylene blue). Main changes observed after immunisation. a) Reactive secondary lymphoid and primary lymphoid follicle (H&E-methylene blue 10×20). b) Great size reactive follicle presenting a salient germinative centre and narrow lymphocyte crown (H&E-methylene blue, 10×20). c) Middle size secondary reactive follicle with well-contoured crown (H&E-methylene blue, 10×20). d) Secondary lymphoid follicle with great germinative centre and narrow lymphocyte crown in course of fibrosis depicting an evolutive process (H&E-methylene blue, 10×20). e) Comparative non-reactive primary and secondary follicles in spleen provided from non-immunised, healthy rabbits (H&E-methylene blue, 10×20 left; 10×40 right).

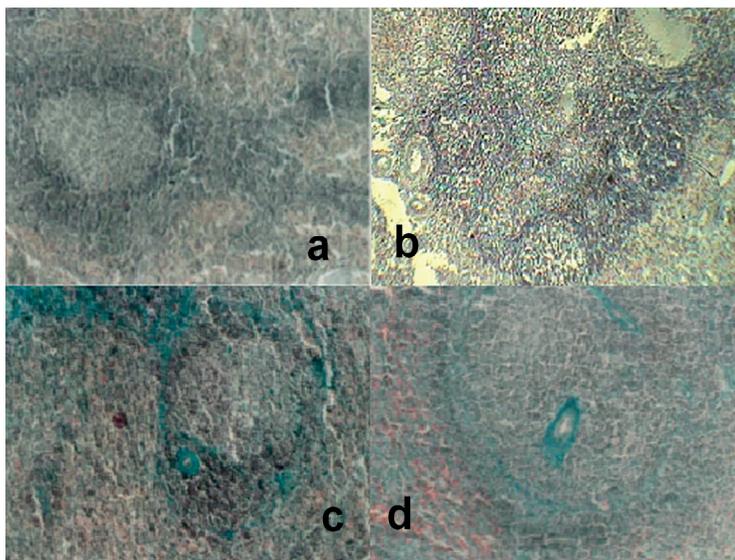


Figure 5: Rabbit lymph nodes. Main changes observed after immunisation. a) Centred secondary gastric reactive lymph node Haematoxylin – Eosin - Methylene blue (H&E-methylene blue, 10×20). b) General view: gastric lymph node - Secondary lymphoid follicle (H&E-methylene blue, 10×20). c) Portal lymph node: secondary lymphoid follicle (H&E-methylene blue, 10×20). d) Secondary lymph node follicle with great germinal centre and narrow lymphocyte crown in course of fibrosis depicting an evolutive process: general view (H&E-methylene blue, 10×20).

experiment due to cellular activation. Comparative analysis of the properdin values' evolution highlighted the effect of vaccine, which undoubtedly stimulated the properdin synthesis, both for primary and secondary antigenic stimulation. The results obtained suggested that in the reception and processing of viral antigens, both cellular elements (lysozyme and properdin) and some non-specific factors were synergistically involved. Our results are confirmed by other studies in this field (Robinson *et al.*, 2002; Le Gall-Reculé *et al.*, 2011; Liu *et al.*, 2012).

Postvaccinal histological observations

The cytoarchitecture examination of the samples collected from the spleen, gastric and portal lymph nodes highlighted characteristic changes in agreement with the postvaccinal immunological response.

Spleen

The white pulp appeared as a diffuse lymphoid tissue, also with primary and secondary lymphoid follicles (Figure 4). This arrangement generated a slight thinning of the parenchyma; connective tissue fibres, which line the adjacent cells, were identified in the free spaces. In all cases, the white/red pulp *ratio* was in favour of the white pulp, in which the lymphoid follicles were dominant, indicating their reactivation. Splenic follicles were enlarged and had a salient germinal centre. Relevant cell proliferation was present in the germinal centre. On the margin of the organ, the capsule was thickened, resulting in exudative perisplenitis. Circulatory disorders, such as congestion and sub-capsular haemorrhages, were present. Moreover, fusions between the white pulp and the red pulp were noted and the red pulp had the aspect of small diffuse islands, irregularly dispersed into the parenchyma.

Portal lymph nodes

In the medullar area we discerned narrow lymphoid cords circumscribed by relatively large lymphatic sinuses and well defined lymphocytolysis; in the cortical area, exudate was observed and lymphoid tissue follicles in course of

activation. We also identified inflammatory processes, morphologically manifested by thickening of the lymph node connective capsule, dilacerations of the connective fibres and the presence of light acidophilic serous exudate with rare inflammatory cells (serous lymphoreticulitis).

Gastric lymph nodes

Reactive secondary follicles are present. A compact cortical showing oedema of the capsule and its partial or total damage was noted. The cortical zone of lymph nodes was densely populated by variable size T lymphocytes, which activate receiving antigens, subsequently triggering and developing the cellular response.

Due to alteration of the vascular permeability in the medullary part, we identified spaces of different size which surrounded the entire circumference of the area. Areas of fibrosis were observed in the form of bluish foci of varying intensity. Between the cortical and medullary zones, the parenchyma of lymph nodes presented numerous visible spaces between cells. The medullary zone was larger than cortical one and occupied by many secondary lymphoid follicles (Figure 5).

CONCLUSIONS

HA antibody concentrations and antibody titre increased significantly and constantly after primary and secondary antigenic stimulation. Comparative analysis of the properdin serum concentration highlighted the effect of vaccine, which stimulated the synthesis of properdin.

Phagocyte index revealed the absence of significant differences between the four experimental periods, but statistically significant differences ($P < 0.05$) were found between the mean value obtained on day 0 and the 3 post-vaccination periods, on days 14, 30 and 60.

Immunological and histological reorganisations were found after vaccine administration in the spleen and lymph nodes, where reactive secondary lymphoid follicles were present.

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