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AN APPROACH TO VIRAL HAEMORRHAGIC DISEASE (VHD): PATHOGENESIS BY HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ASSAY

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ABSTRACT

23 white crossbred rabbits were inoculated with 0.5 ml of 10% liver suspension of VHD diseased animals. In addition, 8 rabbits were kept as negative controls and 4 were placed in contact with the infected animals. Samples of infected organs were taken every four hours in order to check histological changes and immunohistochemical reactivity.
Virological studies were conducted by negative staining immunoelectronmycroscopy. Eventhough symptoms, gross and microscopic lesions did not become evident until 32 hrs p.i., immunohistochemical positivity was recorded from 12 hrs p.i. in liver Kupffer cells. Kidney tubular cells were positive only in rabbits spontaneously dead. Hepatitis was the consequence of viral replication followed by disseminated intravascular coagulation (DIC). Contact rabbits

showed clinical sympthoms starting from 72 hrs p.i.; 3 of them died spontaneously and one survived.

INTRODUCTION

While aetiology, clinical signs, pathological changes and diagnostic methods of VHD are all well known (OIE.1991), little is known about its pathogenesis. The purpose of this preliminary research is closely to examine the pathogenetic mechanisms of VHD. Therefore the organs of experimentally infected rabbits were submited to histopathological, immunohistochemical and immunoelectronycroscopy (IEM) examination.

MATERIALS AND METHODS

EXPERIMENTAL INFECTION

35 white crossbred clinically healthy, seronegative, 3 month old rabbits, whose body weight ranged from 2 to 2.5 kg, were placed in previously cleaned and disinfected single cages. Twenty-three of them were inoculated intramusculary (right leg) with 0.5 ml of a 0.6% cell free liver suspension obtained from diseased animals, kept frozen at -20 in a 50% glycerol solution and containing approximately $10^{5.6}$ $L.D._{50}$ of VHD virus (VHDV). Four rabbits were inoculated with a PBS solution as a placebo and placed in contact with the infected animals. The remaining 8 rabbits, held as negative controls, were kept separate from the others. The

body temperature of each animal, clinical and pathological changes were recorded regulary. The first infected rabbit was sacrificed 8 hrs p.i. and the others at 4 hrs intervals. The animals were deeply anesthetized with ether and killed bloodless.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EXAMINATION

Samples of lung, trachea, heart, liver, spleen, skeletal muscle, brain and kidney were collected during necropsy and fixed by immersion in 10% buffered formalina. After fixation they were rinsed in tap water, dehydrated in ethyl alcohol, embedded in paraffin wax, cut at 4 um and stained with haematoxilin-eosin (HE) and alcian blue-PAS (pH 2.5). In addition, an immunohistochemical staining was carried out using a direct peroxidase procedure. A monoclonal antibody (MAbs) (1H8), from our laboratory in Brescia (Capucci et al.1991), anti-VHDV conjugated with peroxidase (Johnstone and Thorpe, 1987), was used at a 1:400 dilution.

ELECTRON MICROSCOPY EXAMINATION

Negative staining IEM was carried out on liver homogenates (10% w/v) previously clarified, incubated with an optimal dilution of a pool of specific MAbs for 1 h at 37°C and finally ultracentrifugated in Arefuge Beckmann at 10.200 g for 15 min (Capucci et al.1991). A 2% solution of phosphotungstic acid (NaPT) (pH6.7) was used for staining. Samples

observation was carried out using a TEM Philips operating at 80 KV at 25.000x.

RESULTS

Hypertermia was scored 20 hrs p.i. (Tab.I).
At 24-28 hrs p.i.clinical symptoms were characterized by slightly depression. From 32 to 48 hrs p.i. symptoms of excitation and sudden death. Specific pathological changes, as observed in natural cases, were evident starting from 32 hrs p.i.. These changes affected many organs with circulatory and degenerative disturbances (Tab.I). The contact rabbits showed clinical symptoms and hypertermia starting from 72 hrs p.i.. Three (3) rabbits died spontaneously between 76 and 120 hrs p.i. and one survived.

HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL EXAMINATION

From 16 to 28 hrs p.i., microscopic lesions were characterized by hydropic degeneration of liver cells and by hyperemia of lungs, spleen and heart (Tab.II). In the following hours disseminated foci of leucocyte infiltration were present in the liver together with hyaline degeneration, necrosis of hepatocytes and distruption of the synusoid walls. Depletion of lymphoid follicles was observed in the spleen. Hyperemia of lung, trachea and spleen increased in intensity. Focal haemorrhages and fibrinous thrombi in the capillaries as a characteristic feature of disseminated intravascular coagulation (DIC) were also

present. The brain showed moderately swollen neurons, oedema and scattered foci of neuronophagia.
Spontaneously deceased animals always showed typical microscopic lesions (Tab.II). PAS stained liver sections showed early loss of positive material (most probably glicogen) from the periphery of the lobule. Hyaline thrombi of capillaries were also strongly PAS positive.
By immunoperoxidase test Kupffer cells showed a positive reaction as early as 12-20 hrs p.i.. This reaction was characterized by little brownish vacuolated and granular precipitates in the cytoplasma. At 20 hrs p.i. Kupffer cell positivity was clearly evident (Tab.II). At 24 hrs p.i. some hepatocytes also reacted in a positively manner. In the subsequent hours the positivity of Kupffer cells gradually diminished and finally disappeared. At 40 hrs p.i., most of hepatocytes were necrotic and appeared strongly positive. The intensity of the immunoperoxidase reaction was correlated with the score of histological changes. In addition, weak positivity was observed in the cytoplasma of kidney tubular cells at 44 hrs p.i. (Tab. II). No other organs resulted positive.
In the deceased contact rabbits hepatic and kidney cells were also clearly positive.
The 8 control rabbits proved to be negative in all tests carried out.

ELECTRON MICROSCOPY

VHDV particles in liver homogenates were observed in rabbits N°4 and 5 as well as in all the others starting from rabbit

N°8. The IEM examination revealed a clumping of VHDV particles in large aggregates.

DISCUSSION

VHD was reproduced in all experimentally infected rabbits. The disease evolution varied considerably among them and one conctac was able to recover. These results seemed to indicate differences in the evolution of the disease related to individual susceptibility.

Viral antigen was first detected in the liver 12 hrs p.i. but specific gross and microscopic liver lesions were detectable only 32 hrs p.i. The Kupffer cell positivity preceded the hepatocyte positivity. This suggests that probably the virus does not replicate massively in Kupffer cells, but reaches the hepatocytes by passive transfer. Viral replication gives rise to hepatitis and liver necrosis leading to viremia and this in turn to immunohistochemical positivity of kidney tubular cells.

Liver IEM confirmed the presence of viral particles antigenically referible to VHD. Disagreement between IEM and immunohistochemical assay in samples Nr. 6 and 7 could be related to lower sensibylitity of the former method.

In the advanced stages of the disease, we observed, as other previously did (Marcato et al., 1988), mycrothrombi in the capillaries of many organs (DIC feature). This lesion seems to be responsable for the haemorrhagic picture of VHD. Our results demonstrate that VHD infection could develop not only by a rapid viral multiplication and extended liver necrosis (together with the outburst of DIC mechanism) but also by a slower viral multiplication which causes focal

liver damage. This second scheme is apparently compatible with the activation of immune system, recovery and survival of the animals.

In conclusion, disease evolution, survival rate and prognosis depend chiefly on the extension of liver damage which in turn is correlated with the individual rabbit resistence.

REFERENCES

1 Buonavoglia C., L.Di Trani,L. Di Pasquale, T. Tirani, F.M. Ruggeri, D. Galassi. 1988. Sui recenti episodi di mortalità nei conigli in Italia. Nota preliminare. Sel. Vet.29 (10): 1509-1510.

2 Capucci L., Scicluna M.T., Lavazza A. 1991. Diagnosis of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev. sci. tech. Off. int. Epiz. 10 (2) : 347-366

3 Johnstone A., R.Thorpe. 1987. Immunochemistry in practice (II Edition). Oxford. Ed. Blackwell Scientific Pubblications.

4 Kozma C., W. Macklin, L.M. Cummins, R. Mauer. 1974. Anatomy, Phisiology, and Biochemistry of the rabbit, pg 57 in "The biology of laboratory rabbit", Weisbroth S.H., R.E. Flatt, A.L. Kraus, Ed. Academic Press, New York, London 57.

5 Liu S.J., H.P. Xue, B.Q. Pu, N.H. Quian. 1984. A new viral disease in rabbits. Anim. Husbandry & Vet. Med. 16 (6):253-254.

6 Marcato P.S., C. Benazzi, G. Vecchi, L. Della Salda, P. Simoni, P. Aiello, G. Tumino. 1988. L'epatite necrotica infettiva del coniglio. Profilo patogenetico di una

nuova malattia emorragica. Coniglicoltura 25 (9): 59-64.

7 OIE (Office internatinal des epizooties). 1991 :Viral haemorragic disease of rabbits and european brown hare syndrome. Rev. sci. tech. off. int.Epiz.,10 (2): 263-529

6 Tijessen P. 1985. Practice in theory of enzyme immunoassays. Ed. Elsevier Amsterdam.

RABBIT N.	HOURS POST INFECTION 0	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64	68
1	39.4	39.7															
2	39.5		39.7														
3	39.3			39.5													
4	40.0				41.2												
5	42.0					39.5											
6	39.3						39.5										
7	39.7							40.9									
8	39.2								41.2								
9	39.2									40.8							
10	39.7										41.0						
11	39.5											41.2					
12	39.7												41.5				
13	39.3								38.0								
14	40.0													41.6			
15	39.4											40.5					
16	39.3														40.6		
17	39.0							41.0									
18	39.4												38.0				
19	39.9											38.0					
20	39.5											39.6					
21	39.8														41.5		
22	39.8															41.5	
23	40.3										39.0						

TABLE I - TEMPERATURE VALUES (SINGLE CHECK) BEFORE AND AFTER INFECTION TOGETHER WITH THE ONSET OF SIMPTOMS AND LESIONS.

ONSET OF SYMPTOMS: MILD RESTLESSNES - SNEEZING

ONSET OF TYPICAL SYMPTOMS AND GROSS LESIONS

39.1 TEMPERATURE OF SPONTANEOUSLY DEAD RABBITS

RABBITS No	HRS p.i.	HISTOLOGY				IMMUNOHISTOCHEMISTRY		
		LIVER	SPLEEN	KIDNEY	DIC	KUPFER CELLS	HEPATO CYTES	KIDNEY CELLS
1	8	–	–	–	–	–	–	–
2	12	–	–	–	–	±	–	–
3	16	+	–	–	–	–	–	–
4	20	++	–	–	–	+	±	–
5	24	++	–	–	–	+	+	–
6	28	++	–	–	–	+	+	–
7	32	++	–	–	–	+	+	–
17*	32	+++	++	++	+	–	+	+
8	36	+++	+	+	–	–	+	–
13*	36	+++	++	++	+	–	+	+
9	40	+++	++	++	+	–	+	–
10	44	+++	+	+/++	±	–	+	+
23*	44	+++	++	++	+	–	+	+
11	48	+++	++	++	+	–	+	–
15*	48	+++	++	++	+	–	+	–
19*	48	+++	++	++	+	–	+	+
20*	48	+++	++	++	+	–	+	+
18	52	+++	++	++	+	–	+	–
12	56	+++	+	+	+	–	+	±
14	60	+++	+	+	+	–	+	±
16*	60	+++	++	++	+	–	+	+
21	64	+++	+	+	+	–	+	±
22	68	+++	++	++	+	–	+	±

HISTOLOGY

LIVER: + RES activation
++ small degenerative or and necrotic foci
+++ extended necrosis

SPLEEN: + lymphoid depletion
++ lymphoid depletion and congestion

KIDNEY: + hydropic swelling of tubular epithelium
++ heavy tubular and glomerural lesions

CIRCULATORY SYSTEM (DIC):
+ presence of hyaline thrombi

IMMUNOHISTOCHEMISTRY:
+ positive reaction
± low positive reaction

TABLE II - HISTOLOGICAL AND IMMUNOHISTOCHEMICAL RESULTS OF INFECTED RABBITS

(*) spontaneously dead