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VIRAL HAEMORRHAGIC DISEASE OF RABBITS (VHD): A PATHOGENETIC STUDY UNDER EXPERIMENTAL CONDITIONS. *

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SUMMARY

To study VHD pathogenesis, 23 white crossbred rabbits were inoculated with 0.5 ml of a 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 blood and organs were taken every four hours to check hepatic function, histological changes and immunohistochemical reactivity. Virological and serological studies were carried out on immunoenzymatic (Elisa Sandwich) method. Although symptoms, macro and microscopic lesions were not evident until 32 hrs p.i., immunohistochemical positivity was recorded from 12 hrs p.i. in liver Kupffer cells. Urine and kidney tubular cells were positive only in spontaneously deceased rabbits. Hepatitis was the consequence of viral replication followed by disseminated intravascular coagulation (DIC). Hepatic enzymes were modified together with coagulation parameters. Contact rabbits showed clinical sympthoms starting from 72 hrs. p.i. Three of them died spontaneously between 76 and 120 hrs p.i. and one (1) survived.

INTRODUCTION

Since 1986 an acute and fatal haemorrhagic disease has been observed in Italy. It mainly affects adult rabbits reared in rural farms (Bonavoglia et al.,1988; Cancellotti et al.,

1988). This syndrome recently has been recognized as rabbit viral haemorrhagic disease (VHD) first described in China in 1984 (Liu et al.,1984). A viral aetiology has been demonstrated and the virus (VHDV), on the basis of its biochemical characteristics, has been considered as a member of the Calicivirus family (Ohlinger et al.,1989; Capucci et al., 1990). While aetiology, clinical signs, pathological changes and diagnostic methods of VHD are well known, little is known about its pathogenesis. The purpose of this study is closely to examine the pathogenetic mechanism of VHD.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

A group of 35 white crossbred clinically healthy seronegative 3 month old rabbits of both sexes (31 females and 4 males) whith body weight ranging from 2 to 2.5 kg were placed in previously cleaned and disinfected individual 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, which had been kept frozen at -20°C in a 50% glycerol solution. The solution contained approximately $10^{5.6}$ $L.D._{50}$. Four rabbits were inoculated with a PBS solution as a placebo and were 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 was recorded. Serum blood samples were collected every 4 hours to evaluate the various haematological parameters (hepatic function, coagulation tests and erythrocytes sedimentation rate (ESR). The contacts were controlled for haematological parameters every 12 hrs. An immuno-enzymatic reaction was used to trace virus and antibodies in the sera of both infected and non infected animals. Clinical and pathological changes were recorded at regular intervals. The first infected rabbit was sacrificed 8 hrs p.i. and the others at 4 hrs intervals. The animals were deeply anasthetized with ether and killed bloodlessly.

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HAEMATOLOGICAL STUDIES

Hepatic function was evaluated by the presence and levels of serum enzymatic activities and evaluated at 37°C on Hitachi 737. Adopted methods, for the listed enzymes, were as follows: cholinesterase (CHE) according to Ellman (1961); aspartate aminotransferase (AST) and alanine transferase (ALT) according to International Federation of Clinical Chemistry (IFCC) (Bergmayer et al.,1977; Bergmayer and Horder 1980); gamma glutamiltransferase (GGT) according to Szasz (1969) and lactate dehydrogenase (LDH) according to DGKC (Anon.1970). For coagulation parameters, platelet counts were performed on coulter staker (Kontron), fibrinogen on A. C. L. (I.L. SPA, Viale Monza 338 Milan, Italy), prothrombin time (PT) on A.C.L. and ESR on Vesmatic (Biesse spa -Monteriggioni -Siena, Italy). Although the literature already reports the fisiological values of rabbits (Kozma et al.,1974) we have checked them by examining 16 seronegative clinically healty white crossbreds and the 8 negative control rabbits in experiment for the parameters under consideration.

Data were analysed by non parametric analysis of variance (Kruskal-Wallis test- S.A.S. procedure - Barr et al., 1979). Comparison was carried out among the controls and infected group before and after the appearance of clinical symptoms.

VIROLOGICAL AND SEROLOGICAL STUDIES

For VHDV detection an Elisa Sandwich was set up using as catcher and tracer IgG anti VHDV purified from hyperimmune serum from convalescent rabbits (Tijessen et al.,1985). OPD (ortophenildiamine) was used as a substrate and the colour reaction was read using a Multiscan spectrophotometer (Titertek Multiscan, Flow Lab.), absorbance 492 nm. Samples having an absorbance value three times higher than the negative control value were considered positive for VHD. The

negative and positive controls used were respectively : a liver extract of a seronegative rabbit and of an experimentally infected rabbit.
As serology test an Elisa Inhibition reaction was used with the same catcher and tracer previously described. On the sensitised plate, the serum was incubated contemporarily with a fixed dilution of VDHV positive liver extract (OD approx. 1). Positive and negative sera were added as controls. The titre of the serum was the dilution having an OD value corresponding to 50% of the OD value of the negative serum.

CLINICAL AND PATHOLOGICAL OBSERVATIONS

Temperature and symptoms were scored for every rabbit involved in the experiment; pathological changes were recorded during necropsy.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES

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 (1H8), from our laboratory in Brescia (Capucci et al.1991), anti-VHDV conjugated with peroxidase (Johnstone and Thorpe, 1987), was used in a 1:400 ratio.

RESULTS

Even if we established to sacrifice the animals as often as every four hours, the very rapid course of the disease, as

observed in natural outbreaks, brought to spontaneous death in 7 rabbits. Therefore the checking protocol resulted slightly modified (Tab.I and II, Fig.1).

CLINICAL AND PATHOLOGICAL REPORTS

Symptoms were almost entirely absent until 28 hrs p.i., afterwards the animals appeared slight depressed and frequently sneezed. Four hours later one rabbit (No.17) showed symptoms of excitation and died in a few minutes. From 32 to 48 hrs p.i. another five rabbits died spontaneously showing the typical symptoms (Tab.I). From 48 to 68 hrs p.i. clinical symptoms were evident. Only 2 rabbits had a haemorrhagic foamy discharge from the nostrils. One rabbit died 60hrs p.i. Hyperthermia was scored 20 hrs p.i. in rabbit No 4. At the necropsy it's showed an intensification of the liver interlobular network. Specific pathological changes, as observed in natural cases, were evident starting from 32 hrs p.i. These changes affected many organs appearing as circulatory and degenerative disorders (Tab.I). Trachea, lung, kidney and spleen presented haemorrhagic lesions; the liver appeared yellowish-brown in colour, brittle and degenerated, while the urinary bladder was always enlarged and the stomach was full of feed. Poor blood coagulation was also scored and lasted until 68 hrs p.i. The contact rabbits showed clinical symptoms and hyperthermia starting from 72 hrs p.i. Three (3) rabbits died spontaneously between 76 and 120 hrs p.i. The last one (C), showed hyperthermia, severe depression with tremors, jaundice and stopped eating around 130 hrs p.i.. At 160 hrs p.i. his conditions improved and in two days he recovered completly.

HAEMATOLOGICAL FINDINGS

The results are reported in Tabs. II-III.

Thirty-six (36) hrs p.i., AST, ALT and LDH were remarkably altered. Platelet values decreased starting from 20 hrs p.i. while fibrinogen values decreased in 3 rabbits and increased in 12 (Tab.II). ESR was almost mantained within the normal ranges (Kozma et al.,1974) and GGT value was modified starting from 44 hrs p.i. The clotting time at 20 hrs p.i. was prolonged and in some instances its value was so high as to be unmeasurable. Alterated values were scored in only one contact rabbit (A).

VIROLOGICAL AND SEROLOGICAL FINDINGS

The results of these tests are reported in Tabs. III-IV. At 20 hrs p.i. liver extraction scored positive for the antigen. Virus appeared in blood 36 hrs p.i. and was detected always at death (Tab. III). Seroconversion was detected in the contact rabbit " C" 72 hrs p.i., and antigen positivity in 3 of 4 contacts 96 hrs p.i. (Tab.III). In 2 infected rabbits low levels of antibodies were scored after 60 hrs p.i. (Tab.IV) while faeces and nasal discharges always scored negative for presence of the virus. Urine of sacrificed rabbits became clearly positive for virus presence 40 hrs p.i..

HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES

From 16 to 28 hrs p.i., microscopic lesions were characterized by hydropic degeneration of liver cells, starting at the liver periphery of hepatic lobules (Tab. V), and by mild hyperoemia of lungs, spleen and heart. In the following hours, disseminated foci of leucocyte infiltration were present in the liver together with hyaline degeneration and necrosis of hepatocytes. Very often the synusoid wall disappeared and hepatocytes projected into their lumen. Depletion of lymphoid follicles was observed in the spleen.

Hyperemia of lung trachea and spleen increase in intensity. Focal haemorrhages and fibrinous thrombi in the capillaries as a characteristic feature of disseminated intravascular coagulation (DIC) wer also present. The brain showed moderately swollen neurons, oedema and scattered pictures of neuronophagia.

Spontaneously deceased animals always showed typical microscopic lesions (Tab.V). 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 small brownish vacuolated and granular structures in the cytoplasma. At 20 hrs p.i. Kupffer cell positivity was clearly evident (Tab.V and Figure 2). At 24 hrs p.i. some hepatocytes also reacted in a positive manner. In the subsequent hours the positivity of Kupffer cells gradually diminished and in the end disappeared. At 40 hrs p.i., most of hepatocytes were necrotic and appeared strongly positive (Figure 3). The intensity of the immunoperoxidase reaction was correlated with the recording of histological changes. In addition, weak positivity was observed in the cytoplasma of kidney tubular cells at 44 hrs p.i. (Tab. V). No other organs appeared positive.

In the deceased contact rabbits hepatic and kidney cells were also clearly positive.

The 8 control rabbits resulted negative in all tests made .

DISCUSSION

VHD was reproduced in all inoculated animals as confirmed by the presence of the virus in the blood stream and in the examined organs. Eight of them, as observed in natural

outbreaks, died spontaneously, mostly around 48 hrs p.i. The rabbits used in the experiment were representative of a homogeneous group (same breed, age, weight, inoculum and management conditions); but the results indicate that the differences in the evolution of the disease were related to individual susceptibility. The viral antigen was first detected 12 hrs p.i.in the Kupffer cells and specific macro and microscopic lesions were detectable only 32 hrs p.i. The Kupffer cell immunohistochemical positivity at 12 hrs p.i. could be the consequence of the hepatic virus clearance. This mechanism takes the virus from the blood early during the infection, as observed by Mims (1987). In fact, viremia was not seen with conventional tests until 32 hrs p.i. The Kupffer cell positivity preceded the hepatocyte positivity. This suggests that the virus probably does not replicate to large extent in Kupffer cells, but reaches hepatocytes through passive transfer. After this time, many Kupffer cells histologically disappear. In the liver, viral replication gives rise to hepatitis and liver necrosis which leads to viremia. The severe liver necrosis seems to be followed by the prompt release of mediators, like thromboplastin, that are recognized as circulating coagulation factors (Figure 4). The above mentioned factors could be the primer of the DIC mechanism. DIC is characterized by fibrin deposits in the microcirculation, secondary breakdown of the related thrombi and diffuse bleeding (Figure 4) (Morfini et al., 1981). In the advanced stages of the disease, we observed, as others did (Marcato et al.,1988), in both experimentally and spontaneously dead rabbits, microthrombi in the capillaries of many organs. DIC seems to be responsible for the haemorrhagic picture of VHD. These lesions are aggravated by the decrese of fibrinogen values and by low platelet counts in some individual, as well as by the increase of fibrinogen in others. That latter paradoxal result seems to be due to secondary fibrinolisis. This mechanism caused a large amount of fibrinogen degradation

products (FDP) which, using standard sampling methods, are probably analitically not differentiable from fibrinogen molecules (Godal et al.,1984). Between the other parameters, the ESR value was not modified owing to the viral aetiology and the mild inflammatory character of VHD. On the contrary, liver enzymes were grossly modified. Their increase is the expression of severe liver damage with main involvement of mitochondrial structures. The enzyme values do not increase simultaneously with the first hepatic lesion and their concentration in the peripheric blood remains low until liver necrosis attains a severe grade. The presence of the virus in the urine is detectable mainly in the deceased rabbits and appears to be the direct consequence of viremia. The kidney tubular cells immunohistochemical positivity was also the consequence of secondary viremia. Moreover, our results demonstrate that VHD infection develops following two different pathogenetic evolution schemes. The first is characterized by rapid viral multiplication, extended liver necrosis followed by the outburst of the DIC mechanism. DIC seems to be responsible as for the haemorrhagic lesions as for the sudden death of the animals. The latter scheme is characterized by a slower viral moltiplication followed by focal liver damage (focal necrosis). This scheme is apparently compatible with the activation of an effective defence mechanism and particularly with the activation of the immune system (antibodies). This complex of reactions preceded by hypertermia within about 12 hrs makes possible the recovery of some more resistant animal, as observed in our experiment in the contact rabbit C. Therefore surviving rabbits may also be found in live spreading of VHD.

In conclusion, our study confirmed that VHD is a severe and contagious disease affecting rabbits (only one contact survived).

Its evolution, survival percentage and prognosis depends chiefly on the extent of liver damage which in turn is correlated with the individual rabbit resistence.

The extension of hepatic damage is well identify by checking the blood level of hepatic enzymes.
Our results confirms Marcato et al.(1988) and House et al. (1990) opinion, i.e.: VHD is a form of severe liver necrosis rather than a simple haemorrhagic syndrome.

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RABB. N.	HOURS POST INFECTION															
	0	8	12	16	20	24	28		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 N°	HS/p.i.	PLT x 10^3/mm^3	P.T./sec.	FIBRINOGEN mg/dl	CHE U/l	AST U/l	ALT U/l	GGT U/l	LDH U/l	ESR
CONTROLS $\bar{x}$	0	414	8	70	828	30	46	6	645	2
Std Error		49	0,32	16	35	3	3	0,60	107	
1	8	574	N.D.	N.D.	755	22	36	6	502	2
2	12	481	N.D.	N.D.	723	44	25	10	456	2
3	16	962	N.D.	N.D.	624	67	48	6	790	2
4	20	400	16	24	544	38	47	9	146	2
5	24	548	8	58	644	24	34	7	634	2
6	28	227	8	60	790	31	41	3	167	2
7	32	271	N.D.	N.D.	627	53	62	4	281	2
17*	32	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.
0-32 h $\bar{x}$		494	10	47	672	39	41	6	425	
Std Error		92	2	11	32	6	4	0,95	91	
8	36	15	8	58	727	2150	500	6	3500	2
13*	36	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.
9	40	132	8	69	1023	3200	2800	27	33500	2
10	44	308	9	105	1053	23200	4650	108	22550	2
23*	44	84	8	103	939	29200	33500	123	28300	2
11	48	166	U.S.	U.S.	894	1250	250	10	1000	2
15*	48	84	8	5	1181	53950	4800	42	49900	2
19*	48	N.D.	N.D.	N.D.	1286	34400	2850	76	27550	2
20*	48	167	9	122	1247	32650	53500	57	25750	2
18	52	44	9	98	1014	29200	4750	3	40100	2
12	56	140	7	199	1775	16000	3550	32	12100	2
14	60	156	U.S.	U.S.	1439	1750	500	12	2350	2
16*	60	U.S.	9	145	1327	21650	2700	10	22950	U.S.
21	64	U.S.	U.S.	U.S.	714	3700	400	100	5550	2
22	68	174	U.S.	U.S.	854	295	78	N.D.	448	2
36-68 h $\bar{x}$		133***	8	100	1105***	18035***	8202***	46***	19674***	
Std Error		23	0,21	18	78	4445	4157	11	4226	

TABLE II - HAEMATHOLOGICAL RESULTS OF INFECTED RABBITS (*** P<0.001) N.D.=NOT DONE U.S.=UNSUITABLE SAMPLE *=SPONTANEOUSLY DEAD

RABBITS No	HS p.i.	PLT 10^3mm^3	P.T. ses	FIBRINOGEN mg/dl	CHE U/l	AST U/l	ALT U/l	GGT U/l	LDH U/l	ERS mm 1st h	ANTIBODIES DETECTION	ANTIGEN DETECTION
A	24	===	===	===	===	===	===	===	===	===	–	–
	48	===	===	===	===	===	===	===	===	===	–	–
	96	256	8	92	929	11750	650	0	9850	2	–	+
	114	U.S.	19.2	105	2069	U.S.	U.S.	U.S.	U.S.	U.S.	–	+
B	24	230	8.2	54	U.S.	U.S.	U.S.	U.S.	U.S.	U.S.	–	–
	48	===	===	===	===	===	===	===	===	===	–	–
	96	132	U.S.	U.S.	805	19	73	74	U.S.	2	–	+
C	24	===	===	===	===	===	===	===	===	===		
	48	466	8.2	41	470	242	54	8	220	2	–	–
	96	17	17	52	U.S.	U.S.	U.S.	U.S.	U.S.	2	–	+++
D	24	323	8.5	41	U.S.	U.S.	U.S.	U.S.	U.S.	2	–	–
	48	===	===	===	===	===	===	===	===	===	–	–
	72	===	===	===	===	===	===	===	===	===	1:160	–
	96	431	8.0	61	675	19	56	8	309	2	1:160	–
	140	===	===	===	===	===	===	===	===	===	1:160	–

TABLE III- HAEMATOLOGICAL AND VIROLOGICAL RESULTS OF CONTACT RABBITS.

U.S. = Unsuitable sample
=== = Not tested

ANTIGEN DETECTION							ANTI-BODIES
RABBITS No	HS p.i.	NASAL DISCHARGE	FAECES	URINE	LIVER EXTR.	SERUM	TITRE
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	–	–	–	+	+	1:10
22	68	–	–	–	+	+	1:20

TABLE IV - RESULTS OF SEROLOGICAL AND VIROLOGICAL FINDINGS.

(*) = SPONTANEOUSLY DEAD

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

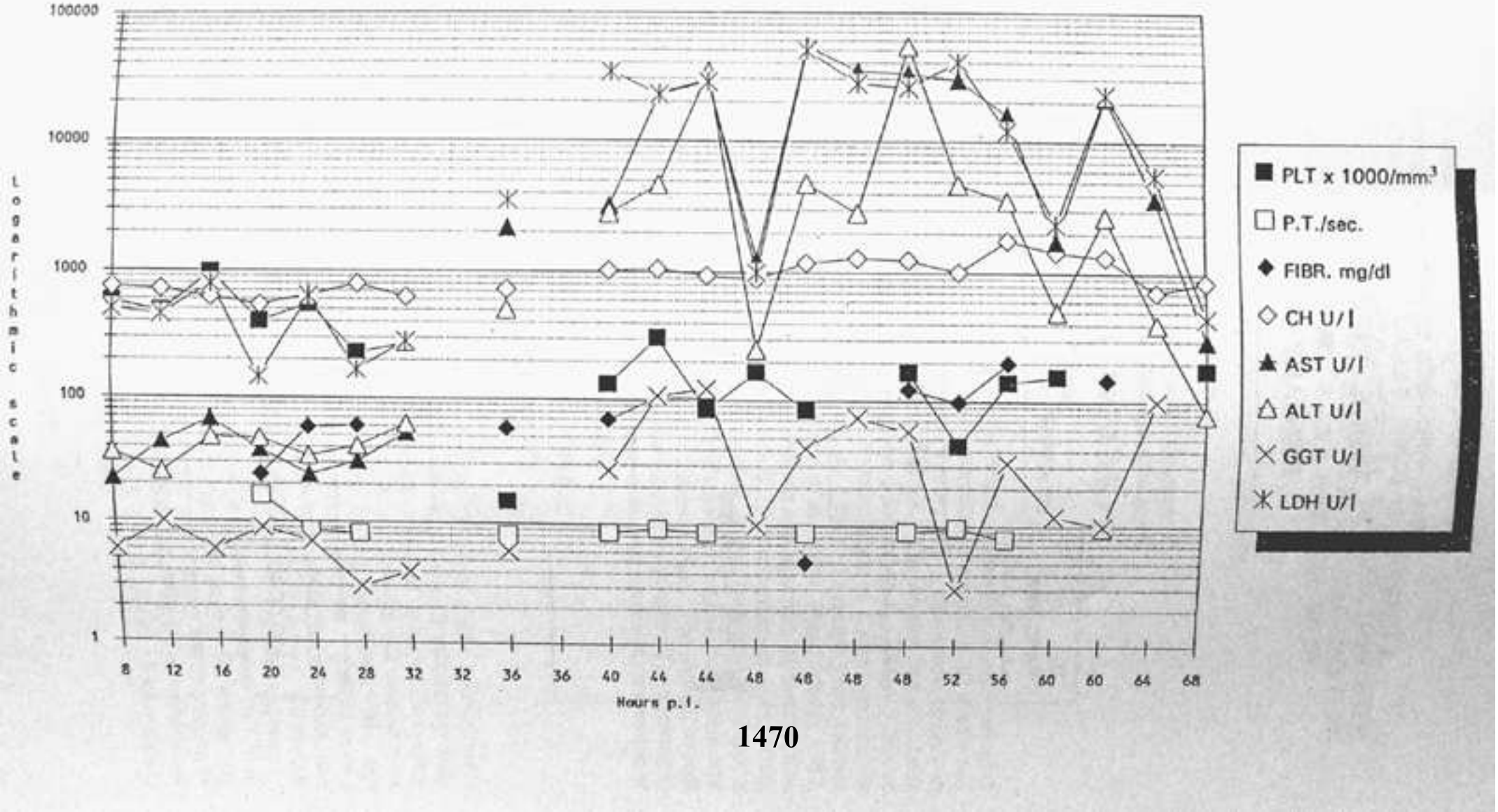
CIRCULATORY SYSTEM (DIC):
+ presence of hyaline thrombi

IMMUNOHISTOCHEMISTRY:
+ positive reaction
± low positive reaction

TABLE V - HISTOLOGICAL AND IMMUNOHISTOCHEMICAL RESULTS OF INFECTED RABBITS

(*) spontaneously dead.

Fig. 1 - Haematological results of infected rabbits



Proceedings 5th World Rabbit Congress, 25-30 July 1992, Corvallis – USA, 1453-1471.

FIG. 4 - HYPOTHESIS OF VHD PATHOGENESIS.