

SUSCEPTIBILITY OF HARES AND RABBITS TO A BELGIAN ISOLATE OF EUROPEAN BROWN HARE SYNDROME (EBHS) VIRUS

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Introduction

During the autumn of 1988 and 1989 high mortality was reported in wild and captive hares in Belgium (Okerman et al. 1989). Affected hares showed suppressed flight reaction and disorientation. Necropsy revealed icterus and pronounced lobular marking of the liver surface. Microscopic lesions were mainly confined to the liver and consisted of periportal or diffuse necrosis of hepatocytes, periportal infiltration of mononuclear cells and hyperplasia of the bile ducts (Uyttebroek et al., 1990). These problems diminished in 1990 and in 1991 no more problems were observed in Belgian hares.

A similar disease pattern has been reported from several other European countries : from Sweden since 1980 (Gavier and Mörner, 1989), from West-Germany since 1986 (Eskens et al., 1987), from Denmark, Italy and France since 1988 (Henriksen et al., 1989, Lavazza and Vecchi, 1989, Morisse et al., 1990) and from Great Britain since 1989 (Chasey and Duff, 1990).

This disease syndrome was grouped under the common heading of "European Brown Hare Syndrome" (EBHS). A non-enveloped, spherically shaped virus of 28 nm was described as the etiologic agent of the disease in most countries (Chasey and Duff, 1990, Eskens et al., 1989, Henriksen et al., 1989, Lavazza and Vecchi, 1989, Morisse et al., 1990). It was called EBHS-virus.

In Italy and in the south of France, a similar disease was observed in wild and in domestic rabbits in exactly the same areas in which EBHS was reported from hares (Lavazza and Vecchi, 1989). Affected rabbits showed 80 to 100 % of mortality. This disease in rabbit was called viral haemorrhagic disease (VHD). VHD is caused by a non-enveloped spherically shaped virus and was characterized as a member of the *Caliciviridae* (Ohlinger et al., 1989). Morphologically, there exists no difference between the VHD-virus and the EBHS-virus. During the Belgian epizootic of EBHS in 1988 and 1989, no such coincidence of a similar disease affecting both rabbits and hares was established. On the contrary, the first outbreaks of VHD in rabbits were reported in 1990, when the occurrence of EBHS in hares had decreased (Peeters et al., 1990).

The aim of the present study was to reproduce EBHS in hares with liver suspensions from Belgian hares which were naturally infected with EBHS-virus and to examine if the Belgian EBHS-virus isolate was able to cause disease in rabbits. Another aim was to examine if the immunity, built up after previous contact with EBHS-virus may protect rabbits against challenge with VHD-virus.

Materials and Methods

Experimental design

Five captive hares H1, H2, H3, H4 and H5 were obtained from a farm where no outbreak of EBHS had been reported. The animals were housed individually. Hare H1 was inoculated subcutaneously (s.c.) with 2 ml of a clarified (10,000 x g) suspension of pooled conjunctivae, lungs and livers, obtained from wild hares showing lesions of EBHS. Hares H2, H3, H4 and H5 were inoculated with a clarified (10,000 x g) suspension of pooled livers, collected from hare H1 and from two naturally infected captive hares which died from EBHS. Diagnosis of EBHS was based on histopathological liver lesions. Hares H2 and H3 were infected s.c. with 2 ml of clarified liver suspension, hare H4 with 2 ml of clarified and filtered (0.45 µm) liver suspension, whereas hare 5 was inoculated orally, intranasally and onto the conjunctivae with 2 ml of the same material (Table 1). Animals were observed daily. Blood was sampled from all hares at inoculation and at euthanasia (hare H1) or one and three weeks p.i. (hare H5).

Table 1. Experimental design and clinical observations

Species	Animal nr	Inoculum	Route of inoculation	Clinical signs	
				Fever (days p.i.)	Death (days p.i.)
Hare	H1	EBHS	s.c.	ND	Yes (6)**
	H2		s.c.	ND	Yes (2)
	H3		s.c.	ND	Yes (3)
	H4		s.c.	ND	Yes (5)
	H5		o.n. + conj	ND	Yes
Rabbit	R1	EBHS 1st inoculation	s.c.	No	No
	R2		s.c.	No	No
	R3		s.c.	No	No
Rabbit	R1	VHD 2nd inoculation*	s.c.	No	Yes (2)
	R2		s.c.	Yes (2)	Yes (4)
	R3		s.c.	Yes (3)	Yes (7)
Rabbit	R4	VHD	s.c.	No	Yes (2)
	R5		s.c.	No	Yes (2)
	R6		s.c.	Yes (2)	Yes (3)
	R7		s.c.	Yes (2 + 3)	Yes (5)
	R8		s.c.	No	Yes (2)
	R9		s.c.	No	Yes (2)
	R10		s.c.	Yes (3)	Yes (4)

s.c. : subcutaneous, o.n. : oronasally, conj. : on the conjunctivae, ND : not done

* second inoculation : 4 weeks after the first inoculation

** euthanasia 6 days p.i.

Ten seronegative rabbits R1 to R10 were inoculated s.c. either with VHD-virus or with material collected from hares affected by EBHS (Table 1). Rabbits R1, R2 and R3 were infected with 2 ml of the same material as the one used in hares H2 and H3. Four weeks later, the same rabbits were challenged with 10³ LD₅₀ VHD-virus. This VHD-virus was isolated during the Belgian-outbreak of VHD in 1990. Rabbits R4 to R10 were injected with 2 ml of the same VHD-virus. Body

temperature was taken daily from all rabbits until day 5 p.i. Blood was sampled at the time of inoculation and four weeks later. Liver samples were subjected to the haemagglutination test in order to show the presence of VHD-virus.

Pathology

Necropsy was performed on all hares after natural death or euthanasia. Portions of conjunctivae, trachea, lungs, liver and kidneys were fixed in 10 % buffered formalin (v/v) and processed for histopathology according to standard paraffin embedding and haematoxylin and eosin staining techniques.

Electron microscopy

A 5 % liver suspension (hare H4) was clarified at 5,000 x g for 30 minutes. The homogenate was layered on a cushion of 30 % (w/w) sucrose and pelleted at 200,000 x g for 1.5 hour. The pellet was resuspended in phosphate-buffered saline (PBS) by ultrasonic treatment during 2 x 10 seconds (MSE 150 Watt Ultrasonic disintegrator). After clarification, the sample was centrifuged for 17 hours at 200,000 x g in a 30 to 55 % linear glycerol potassium tartrate density gradient (Objeski et al., 1974). Bands visualized by light scattering were collected, dialyzed against PBS and used directly for electron microscopy (EM). Specimens were prepared using 200 mesh formvar coated grids and stained with 2 % potassium phosphotungstate, pH 6.1. They were examined in a Zeiss EM 95-2 transmission electron microscope at an instrumental magnification of 28,000.

Immuno-electron microscopy (IEM) was performed with preinoculation and convalescent sera from hare H5. Equal volumes of serum and purified virus preparation were mixed and incubated at 37 °C for 2 hours and 4 °C overnight. After centrifugation at 10,000 x g for 30 minutes, the pellet was resuspended in PBS and prepared for EM as described above.

Haemagglutination and agar gel precipitation test

Presence of VHD-virus in liver suspensions was detected by an haemagglutination test using human type O red blood cells as described before (Peeters et al., 1990). EBHS-virus was detected by agar gel precipitation. A 20 % liver suspension was prepared in PBS. The suspension was centrifuged at 10,000 x g during 10 minutes. The supernatant was collected and treated with freon. The aqueous phase was centrifuged at 14,000 x g during 10 minutes. The supernatant was used as antigen. An anti-EBHS-virus convalescent serum obtained from an experimentally infected hare was used as antiserum.

Serology

Presence of serum antibodies against VHD-virus and EBHS-virus (hare H5 and rabbits R1, R2 and R3) was shown by blocking ELISA performed in the Istituto Sperimentale della Lombardia e dell Emilia - Brescia, Italy (Capucci et al., 1991).

Biochemistry

Biochemical and enzymatic determinations of total bilirubine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltranspeptidase (gamma-GT) were performed with the sera of the experimentally infected hares H1 and H5.

Results

Clinical observations

The clinical results obtained during the different experiments are summarized in Table 1. No clinical signs were observed in hare H1 until six days p.i.. Then a mucopurulent discharge became apparent from the nostrils. The conjunctivae took a yellowish shade. At that moment the animal was sacrificed. Hares H2 and H3 died two and three days p.i. respectively. A suppressed flight reaction and an unstable gait were noticed 8 to 12 hours prior to death. Hare H4 died five days p.i. A suppressed flight reaction was the only clinical sign which was observed during the two days preceding death. Hare H5 showed a liquid discharge from the nostrils on the second and third day p.i. Moreover, some fibrinous material was present in one eye 3 days p.i. This hare survived the experimental infection.

Rabbits R1, R2 and R3 survived the inoculation of EBHS-virus. No changes in body temperature were established. Yet, after challenge with VHD-virus rabbits R1, R2 and R3 died 2, 4 and 7 days p.i. respectively. Two of them showed fever p.i. Rabbits R4 to R10 died two to five days following inoculation of VHD-virus. Two of them showed rise of body temperature on the second and third day p.i.

Pathology

At necropsy, hare H1 showed icteric discolouration of subcutis, aorta intima and subcutaneous connective tissue. Faint petechial hemorrhages and hemorrhagic streaks were observed in the thigh and in the dorsal subcutis. A muco-purulent exsudate was present in the bronchi. The liver showed minimal gross lesions, with the exception of an increased marking of interlobular septa. Histopathology revealed infiltration of lymphocytes in the portal area, extensive vacuolation of periportal hepatocytes and proliferation of bile ducts. The apical lung lobes showed purulent broncho-pneumonia. Desquamation of bronchiolar epithelial cells was detected in the diafragmatic lung lobes.

Necropsy of hares H2, H3 and H4 revealed similar pathological changes. A moderate icteric discolouration was found in the subcutis and in the aorta intima. The liver showed congestion and was brittle on palpation. The lobular marking was pronounced. Parenchymatous liver changes were diffuse and severe and consisted essentially of vacuolar degeneration and individual necrosis of hepatocytes, beside congestion of the sinusoids. Liver cells were most severely affected in periportal areas. The lungs revealed pinching-off of cytoplasmic droplets from bronchiolar epithelial cells (H2 and H4) and/or a desquamation of the bronchiolar epithelium (H3 and H4).

Electron microscopy

Density gradient centrifugation of clarified liver homogenate from hare H4 showed two closely spaced bands located in the zone with a density of 1.34 g/ml. EM confirmed that both bands contained morphologically identical virus particles. The particles appeared roughly spherical and measured 28-30 nm in diameter. They had an indistinct outline and some were penetrated by stain and revealed an electron-dense centre. IEM revealed immune complexes consisting of large aggregates of widely spaced particles. The virions were surrounded by a fuzzy rim of antibodies. No immune complexes were detected when preinoculation serum was used to perform IEM.

Haemagglutination and agar gel precipitation test

EBHS-viral antigens were detected in the livers of hares H1, H2, H3 and H4 by agar gel precipitation. Precipitation lines were formed with anti-EBHS-virus convalescent serum only and not with serum from uninoculated hares.

VHD-viral antigens were detected with the haemagglutination test in all livers of rabbits that died after experimental infection with VHD-virus. The haemagglutination was inhibited with a hyperimmune anti-VHD rabbit serum, confirming the specificity of the reaction.

Serology

No antibodies against EBHS-virus, nor against VHD-virus were detected in hare H5 nor in rabbits R1, R2 and R3 at the time of inoculation. Three weeks after infection with EBHS-virus, a titer of 640 was found in hare H5 with the EBHS-virus blocking ELISA and a titer of 20 with the VHD-virus blocking ELISA. A titer of 40 was found with the EBHS-virus blocking ELISA in rabbits R1, R2, R3 four weeks after infection with EBHS-virus. Anti-VHD-virus titers were not detected in the latter sera.

Serum biochemistry

At the moment of euthanasia, the concentrations of total bilirubine, AST, ALT and gamma-GT of hare H1 increased twentifold in comparison with the values obtained prior to inoculation. In hare H5, AST showed a three-fold and ALT a four-fold increase one week p.i. In the latter hare, the values of both enzymes became normal again three weeks p.i.

Discussion

The results of the present experiments prove that the European brown hare syndrome can be reproduced in hares but not in rabbits with a Belgian EBHS-virus isolate. After experimental infection of captive hares with the Belgian isolate of EBHS, similar clinical signs were established as those described in Belgian wild hares (Uyttebroek et al., 1990) and in wild and captive hares from other European countries (Chasey and Duff, 1990, Eskens et al., 1989, Henriksen et al., 1989). A terminal altered behaviour consisting of a suppressed flight reaction was the most frequently observed symptom.

Also the histo-pathological changes, predominantly liver lesions, were comparable to the lesions described before (Gavier and Mörner, 1989, Eskens et al., 1987, Eskens and Volmer, 1989, Okerman et al., 1989, Uyttebroek et al., 1990). They were characterized by degeneration of hepatocytes, especially in periportal areas, by hyperplasia of the bile ducts and by infiltration of mononuclear cells. Evidence of a hepatocellular disease was also given by the significant increase of the serum level of alanine aminotransferase (ALT), by the increase of serum bilirubine and by the accumulation of bilirubine in the tissues.

The EBHS-virus isolated from Belgian hares showed a diameter of 28-30 nm and a density of 1.34 g/ml. These characteristics are similar to those of the EBHS-virus (Chasey and Duff, 1990) and of the VHD-virus (Granzow et al., 1989, Nowotny et al., 1990, Ohlinger et al., 1989, Smid et al., 1989). Both viruses are spherical and show regularly distributed cup-shaped depressions at the periphery (Granzow et al., 1989, Nowotny et al., 1990, Ohlinger et al., 1989).

Some discrepancies were reported from the literature about the susceptibility of rabbits to the EBHS-virus. In Italy and in the south of France, affected rabbits were present at the same time

and in the same regions as EBHS-affected hares. The virus isolated from the liver of French EBHS-affected hares was able to kill rabbits after experimental inoculation and was neutralized by VHD-hyperimmune serum (Morisse et al. 1990). These observations are in contrast with those made in Belgium, Denmark, Great Britain, Sweden and West-Germany. In these countries no simultaneous problems were observed neither in rabbits nor in hares. Moreover, our results and the results mentioned by Eskens and Volmer (1989) show that EBHS-virus does not induce clinical signs in seronegative rabbits.

Noteworthy in the present study is the low titer of anti-VHD-virus antibodies found in one hare infected with EBHS-virus. This finding suggests the presence of common antigenic determinants on both EBHS-virus and VHD-virus. Such antigenic relationship between the EBHS-virus and the VHD-virus has been demonstrated by means of monoclonal antibodies (Capucci et al., 1991) : six out of eight monoclonal antibodies which are directed against epitopes of VHD-virus also reacted with epitopes of EBHS-virus.

Low titers of anti-EBHS-virus antibodies were found in rabbits after subcutaneous inoculation of EBHS-virus. It was impossible to conclude whether this was the result of an antigenic stimulation of the immune system by the inoculum itself (without virus replication) or whether it was induced by limited virus replication. Anyhow, this low level of anti-EBHS-virus antibodies did not protect rabbits against challenge with VHD-virus. This lack of protection may be explained by the absence of common neutralizing epitopes. Capucci et al. (1991) have already demonstrated that two out of eight monoclonal antibodies which were produced against VHD-virus and which neutralize VHD-virus, do not react with EBHS-virus.

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Summary

Clinical signs and pathological changes of the European brown hare syndrome (EBHS) were reproduced in hares after subcutaneous inoculation with liver suspensions from Belgian hares, which were naturally infected by EBHS. Electron microscopy established the presence of virus particles post-infection (p.i.). They showed a spherical shape, a diameter of 28-30 nm and a density of 1.34 g/ml. Immuno-electron microscopy identified the virus as the etiologic agent of EBHS. In seronegative rabbits the same virus did not induce clinical signs after subcutaneous inoculation. Although they showed low levels of antibodies against EBHS-virus in the serum four weeks after infection with EBHS-virus, the rabbits were not protected against cross-challenge with the viral haemorrhagic disease (VHD) - virus.