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Pathological study on rabbit haemorrhagic disease in young rabbits

Young-suk Kim, Yong-soon Lee, Kang-moon Seo*, Yeon-hee Lee**, Jae-hak Park***

Department of Veterinary Public Heath, College of Veterinary Medicine, Seoul National University, Suwon, Korea
*Department of Surgery, College of Veterinary Medicine, Kangwon National University, Korea**
*Department of Biology, Seoul Women's University, Seoul 139-774, Korea***
*Department of Laboratory Animal Science, College of Veterinary Medicine, Seoul National University, Suwon, Korea****

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Abstract : We investigated the pathological changes in young rabbits which were experimentally infected with rabbit hemorrhagic disease virus (RHDV). Experimental infection of RHDV was carried out in both thymectomized and non-thymectomized young immature rabbits and adult rabbits. None of young rabbits infected with RHDV died during the experiment. Histologically, single or focal hepatocellular degeneration and necrosis with mild lymphocyte infiltration were observed in the rabbits killed at 30 hours and 5 days PI. Lymphocyte infiltration was more severe at 5 days PI than at 30 hours PI. RHDV antigens were mainly detected in the degenerating hepatocytes adjacent to the infiltrated lymphocytes at 30 hours PI and 5 days PI. In electron microscopical observation, infiltrated lymphocytes in the lesions had large nuclei without cytoplasmic granules and interdigitated with adjacent hepatocytes. It is assumed that infiltrated lymphocytes in hepatic lesions in RHDV infected young rabbits are T-lymphocytes and originate from peripheral lymphoid organs or tissues rather than from thymus.

Key words : RHDV, rabbits, thymectomy.

Introduction

Rabbit hemorrhagic disease (RHD) was first reported in China (Liu *et al*[3], 1984). It has been known that this disease occurred mainly in adult rabbits, while it seldomly occurred in rabbits younger than 2 months old (Park *et al*[4], 1987). However, it is not known why young and immature rabbits are resistant to RHDV infection. Moreover, there has been no report on the histopathological changes of young rabbit infected with RHDV. Possible explanations for the difference between young and adult animals might be the pres-

Address reprint requests to Dr. Jae-hak Park, Department of Laboratory Animal Science, College of Veterinary Medicine, Seoul National University, Suwon 441-744, Republic of Korea.

ence of the thymus and the alteration of viral receptors on the cytoplasmic membrane according to the development.

Because the thymus plays an important role in the immune response, we hypothesized that thymus-derived T-lymphocytes may be involved in the defense of young immature rabbits against RHDV infection. To test this hypothesis, we examined the pathological changes in young normal rabbits and thymectomized rabbits after they were infected experimentally with RHDV.

Materials and Methods

Liver homogenate (20% suspension, HA 10^{18}) of a New Zealand White rabbit, which were experimentally infected with RHDV, was prepared in phosphate buffered saline (10mM, pH 7.2) and used for infection. Eight 6 months old male New Zealand White (NZW) rabbits, weighing 3.0~4. 5kg, were injected with 0.5ml of the hepatic homogenate intramuscularly and they were necropsied soon after death. When four 4 weeks old male NZW rabbits, weighing 0.9~1. 1kg, were injected with 0.5ml of liver homogenate, two of them were killed at 30 hours post infection (PI) and 5 days PI, respectively.

Thymi of the 4 weeks old NZW rabbits were resected according to Waynforth's method (1992). Following to the preparation of artificial respiration by tracheotomy, thoracic incision was carried out. The sternum was incised and retracted from manubrium to the third sternum. The thymus was picked up and separated from connective tissues. When the pleura was ruptured, artificial respiration was performed. After thymus resection, the thoracic cavity was closed after the elimination of remnant air in the cavity.

Two days after total thymectomy, four rabbits were injected with 0.5ml of liver homogenate and rabbits were killed under anesthesia with pentobarbital sodium at 30 hours PI and 5 days PI. Liver, spleen, lung, kidney, heart, thymus, brain and intestines of all rabbits were fixed in 10% phosphate buffered formalin and embedded in paraffin. Tissue sections embedded in paraffin were cut to have 3μm thickness and stained with hematoxylin & eosin (HE).

Sections from the tissues were stained with the direct Avidin Biotin Complex (ABC) immunohistochemical method. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 10 minutes after deparaffinization and rehydration. To block non-specific binding of biotinylated anti-RHDV IgG, the sections were incubated in 1% BSA and 5% skim milk dissolved in PBS for 1 hour. The sections were incubated for 4 hours with biotinylated rabbit anti-RHDV IgG which was made in our laboratory (Park and Itakura[5], 1992). This was followed by washing in PBS with three changes each for 30 minutes and then incubated with ELITE ABC kit (Vector Laboratory, USA) for 100 minutes. After washing the sections in PBS with three more changes each for 15 minutes, peroxidase activity was detected by 10 minutes incubation in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB : Sigma, USA) containing 0.05% hydrogen peroxide, then counterstained with hematoxylin.

Thymocytes were obtained from the thymus of a 4 weeks old rabbit. After washing in PBS with three changes, three guinea pigs were injected intravenously with 1×10^8 of thymocytes. A boost was performed four weeks later after the first injection. Six days later, the sera of guinea pigs were collected and used as rabbit thymocyte antisera. Liver, spleen and thymus of 4 weeks old rabbits were fixed in periodate-lysine-paraformaldehyde at 4℃ for 24 hours. Tissues were processed and embedded following the Whiteland's method (1995). Sections were stained with the direct immunohistochemical method with a little modification.

Normal guinea pig serum (1 : 50) instead of antiserum against thymocyte was used as a negative control. In addition, direct immunohistochemistry was carried out using peroxidase conjugated goat anti-rabbit IgG (GIBCO BRL, USA).

Formalin fixed liver and thymus were washed with PBS overnight and fixed in 1% osmium tetraoxide for 1 hour after being fixed in 2% glutaraldehyde. They were dehydrated and embedded in Quatol 812. After observation of a semithin section stained with 1% toluidine blue, thin sections were cut and stained with uranyl acetate and lead citrate and then examined with an electron microscope (JEM 100CX II, JEOL, Japan).

Results

Adult rabbits : All RHDV infected rabbits died between 36 hours PI and 5 days PI. Some rabbits showed depression, no activity, pallor, droop of the ears, a queer sound cry, convulsion and opisthotonus. Foamy blood discharges from the nostrils were observed from infected rabbits. The liver was mildly enlarged and friable, its surface was rough and discolored, and the cut surface had a distinct lobular pattern. Multiple petechiae or ecchymotic hemorrhage were observed in the lung. The kidney and spleen were severely congested. Multifocal necrosis was observed throughout the hepatic lobule (Fig 1). Hepatocellular necrosis was more severe in Zone I in arcinal structure. Mild infiltration of mononuclear cells was observed in the Glisson's sheath. Degenerating hepatocytes were ballooned. Necrotic hepatocytes were shrunken, occasionally infiltrated with heterophils, and showed karyorrhexis, karyopyknosis or karyolysis. Some karyolytic nuclei lost their basophilicity and showed eosinophilicity. There was severe congestion and heterophilia in the red pulp of the spleen. The lung was markedly congested and there were hemorrhages in the alveoli and interlobular connective tissues. Fibrin thrombi appeared in the capillaries with migration of heterophils (Fig 2). There was severe congestion in the kidneys. Fibrin thrombi were seen in the glomerular capillaries and small vessels in the connective tissues.

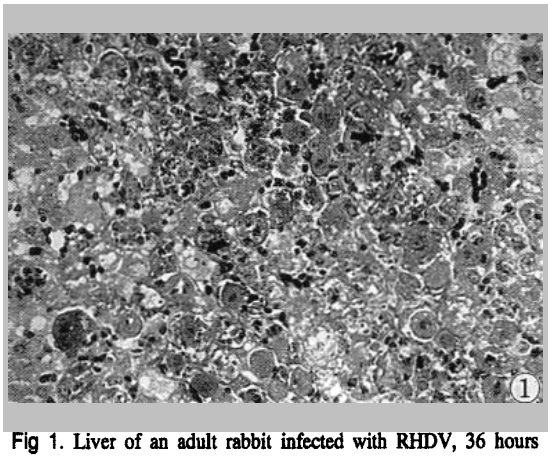

Fig 1. Liver of an adult rabbit infected with RHDV, 36 hours PI. Note ballooning degeneration (arrows) and marked necrosis of hepatocytes. HE× 200.

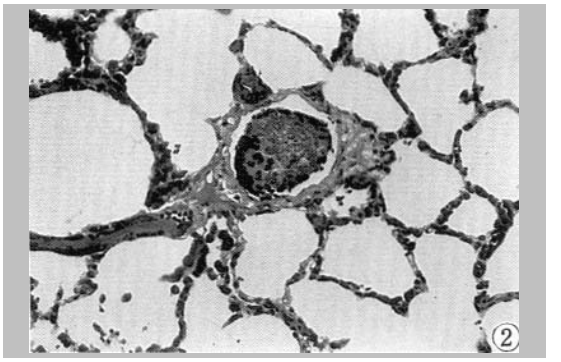

Fig 2. Lung of an adult rabbit infected with RHDV, 36 hours PI. Fibrin thrombi are seen in the small vessel. HE× 300.

Four weeks old rabbits : There was no mortality after RHDV was injected. Both thymectomized and non-thymectomized rabbits showed mild depression. There were not any significant lesions on the surface or the cut surface of the liver. Petechiae were observed in the lung of both groups.

The lesions in the liver of thymectomized and non-thymectomized rabbits were similar. Focal necrosis and lymphocytic infiltration in the necrotic area were seen in rabbit killed at 30 hours PI and 5 days PI (Fig 3). Lymphocytic infiltration was more severe at 5 days PI. Necrotic hepatocytes were shrunken and their nuclei showed pyknosis and karyolysis. Some of the nuclei showed eosinophilicity. Eo-

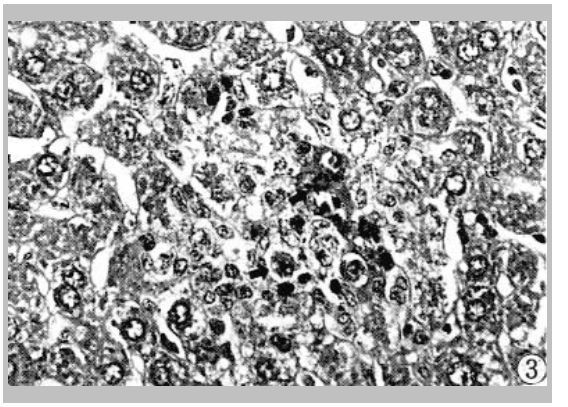

Fig 3. Liver of a young thymectomized rabbit infected with RHDV 30 hours PI. Note single hepatocellular necrosis (arrows) with mild lymphocyte infiltration. HE× 300.

sinophilic globules appeared with high frequency in the sinusoids in the lesions. Lymphocytic and heterophilic infiltration were seen in the necrotic area. Around the infiltrated heterophils, small eosinophilic granules of degranulated heterophils were seen. There wasn't any significant lesion in the spleen, lung and kidney.

The patterns of RHDV antigen distribution in the liver were not different from each other in thymectomized and non-thymectomized rabbits. RHDV antigen was detected in some degenerating and necrotic hepatocytes of the rabbits 30 hours PI and 5 days PI (Fig 4).

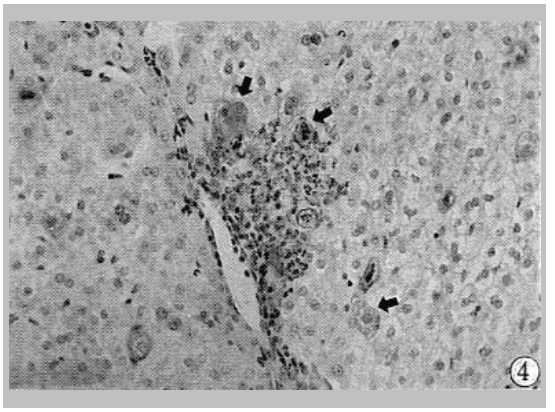

Fig 4. Liver of a young thymectomized rabbit infected with RHDV 30 hours PI. Hepatocytes adjacent to the infiltrated lymphocytes show positive reactions with the anti-RHDV IgG (arrows). ABC immunostaining. × 400.

Under an electron microscope, infiltrated lymphocytes in the liver of 4 weeks old rabbits had increased N/C ratio and ribosomes in the cytoplasm than those in normal adult rabbits. Lymphocytes were linked to the membrane of degenerating hepatocytes and some of them were interdigitated with hepatocytes. The interdigitation was characterized by close adhesion between lymphocytes and hepatocellular membrane or contact in distance (Fig 5).

Most of thymic cells of thymus showed positive reaction at the cell membrane in immunohistochemistry with guinea pig anti-rabbit thymocyte antiserum. Lymphocytes, infiltrated in the area of focal hepatocellular necrosis, showed intense antigen positive reaction, and some Kupffer cells showed antigen positive reaction (Fig 6).

When guinea pig anti-rabbit thymocyte antiserum was replaced with normal guinea pig serum, liver, thymus and spleen did not show a positive reaction. Infiltrated lymphocytes in the area of focal hepatocellular necrosis showed no positive reaction with goat anti-rabbit IgG.

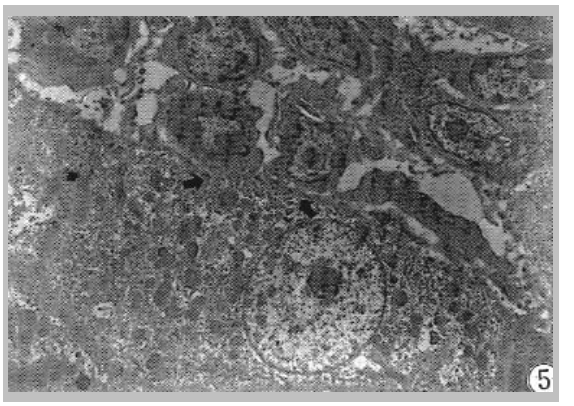

Fig 5. Liver of a young thymectomized rabbit infected with RHDV 30 hours PI. Lymphocytes are interdigitated with hepatocytes (arrows). An electromicrograph. × 3,000.

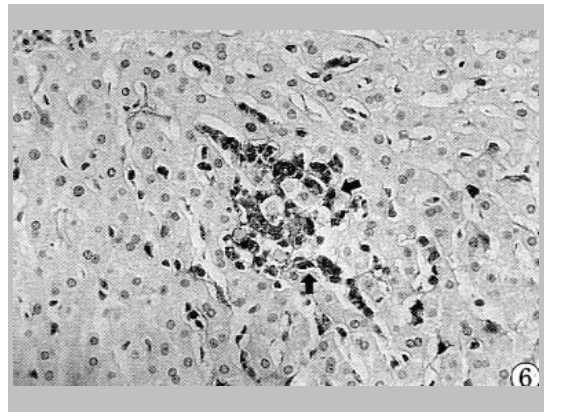

Fig 6. Liver of a young rabbit infected with RHDV 30 hours PI of non-thymectomized group. Lymphocytes near the necrotic hepatocytes showed positive reaction to the anti-thymocyte antiserum (arrows). ABC immunostaining. × 200.

Discussion

RHD is characterized by sudden death at 2 or 3 days PI in experimentally or naturally RHDV infected rabbits. The characteristic histopathologic changes are acute hepatitis and disseminated intravascular coagulation (DIC) in various organs (Park *et al*[5], 1992).

Tizard (1995) observed different effects of thymectomy on neonatal and adult rabbits. The number of circulating lymphocytes drops to a very low level in neonatally thymectomized rabbits, and an animal becomes susceptible to infections as a result of this. In contrast to neonatal thymectomy, surgical removal of the thymus in an adult rabbit produced no immediately obvious result. Based on this observation, we tried to increase the susceptibility of rabbits to RHDV by removing the thymus in young rabbits.

In this experiment, thymus was resected after thoracic cavity was opened from manubrium following the Waynforth's method (1992). Akimaru *et al* [1](1994) suggested median sternotomy from the xyphoid process rather than incision of the cervical portion because of the following problems : 1) The operation field could not be observed properly. 2) Ligation of thymic vessel is not easy. 3) Whole resection of the thymus may be incompleted. However, our previous study showed that Akimaru's method caused extension of the operation time and had shortcomings not found in the Waynforth's method, as Akimaru *et al* [1] pointed out.

Focal hepatocellular necrosis and lymphocytic infiltration appeared in all of 4 weeks old rabbits, irrespective of thymectomy. Thymectomized and non-thymectomized rabbits showed RHDV antigen positive reaction. These results showed that RHDV failed to kill the young rabbits even though it could infect. RHDV antigen was mainly detected in the randomly scattered individual hepatocytes at 30 hours PI. However, it was more frequently detected in the hepatocytes infiltrated with lymphocyte than in the scattered individual hepatocytes. It is assumed that lymphocytes infiltrate for the defense against infection in young rabbits. By the fact that focal hepatocellular necrosis and lymphocytic infiltration occurred irrespective of thymectomy, we assumed that the infiltrated lymphocytes in RHDV infected young rabbits originated from peripheral lymphoid organs, not directly from thymus.

The infiltrated lymphocytes in or around the necrotic and degenerating hepatocytes reacted positively with this guinea pig antiserum anti-rabbit T-lymphocytes. From these results, we suggest that the infiltrated lymphocytes in the lesions of the livers of young rabbits infected with RHDV were of T-cell origin.

Most of the infiltrated lymphocytes in lesions of the livers of young rabbits infected with RHDV had large nuclei and high N/C ratio without cytoplasmic granule when observed under an electron microscope. These lymphocytes, infiltrated in the lesions, surrounded the necrotic and degenerating hepatocytes and seemed to be cytotoxic T-cell rather than NK-cell. These lymphocytes tightly contacted or interdigitated with hepatocytes. We suggest that lymphocytes on contact with hepatocytes remove the virus-infected hepatocytes. Lymphocytes, which infiltrated in the lesions of the livers in thymectomized young rabbits infected with RHDV, seemed to originate from peripheral lymphoid organs rather than from thymus. Because it is unlikely that these would be any effect on the peripheral lymphoid pool by reduced thymic export, in 2 days.

In this study, we found that peripheral lymphocytes played an important defensive role against RHDV infection in young rabbits. In contrast to this, lymphocytic infiltration could not be found at the initial stage of infection in adult rabbits, as reported by Park *et al* (1995). The reason why peripheral lymphocytes cannot play a role against RHDV infection in adult rabbits is under investigation in our laboratory.

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