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Studies on the rabbit viral hepatitis : Immunohistochemical observations

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토끼의 바이러스성 간염에 관한 연구 : 면역조직화학적 관찰

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초록 : 토끼의 바이러스성 간염, 소위 토끼 출혈병의 원인체에 대한 각종 세포주들에 바이러스증식을 유도하고 한편 실험적 감염예에 대한 면역형광항체법과 immunoperoxidase 방법에 의한 원인체의 조직내에 분포상황을 조사하기 위하여 감염된 토끼를 폐사직후 부검하여 간장, 비장, 신장, 폐 및 뇌조직을 절제하여 동결절편하거나 또는 포르말린고정 파라핀 포매 절편을 5~7 μm로 작제하여 면역반응에 공시하였다.

공시된 각종 세포주에 대한 원인체의 세포배양성은 인정되지 않았으며 면역조직화학적방법을 이용한 면역반응에서는 간장에서 강한 양성반응을 보였으며, 비장과 신장에서는 소수예에서 백색수주변 대식세포와 신사구체에서 양성반응을 각각 나타내었다. 그러나 기타 장기에서는 특이한 양성반응이 인정되지 않았다.

ABC immunoperoxidase 방법을 이용한 간장의 포르말린고정 파라핀포매 절편에서 portal triad를 중심으로한 소엽주변부 간세포에서 강한 양성반응을 나타내었으며, 이들 양성반응은 간세포 및 동양혈관세포의 세포질내에 미세과립상으로 미만성 및 세포질주변성으로 관찰되었다. 그리고 감염세포와 비감염세포와의 구별이 명확히 인정되었고 양성반응을 보이는 부위는 H-E 염색상 변성괴사된 간세포에 일치되었다.

이상의 결과에서 본 질병의 표적기관은 간장이며 포르말린고정 파라핀포매 간조직의 immunoperoxidase 방법에 의한 면역조직화학적방법이 본병 진단에 크게 활용되리라 본다.

Key words : RHD, viral hepatitis, immunofluorescence, ABC immunohistochemistry.

Introduction

Rabbit viral hepatitis, so-called rabbit hemorrhagic diseases(RHD) is an acute fatal disease only in rabbits, which is characterized acute hepatitis.[1] After first outbreak of RHD in China since 1984[2], RHD is also out-

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broken subsequently in European countries[3~6] and in south America.[7] Recently, the causative agent has been classified as a candidate calicivirus from the morphology, physicochemical properties, nucleic acid profile, and electrophoretic protein patterns of this virus.[4,6]

The conspicuous pathologic findings were hemorrhages in the lungs, liver, kidneys and heart of naturally infected rabbits. In experimentally infected rabbits, marked lesions were consistently found in the liver.[1,9,10] However, viral pathogenesis in RHD has not been well evaluated until now, although ultrastructural changes in affected tissue were in part examined.[1,9]

This paper describe virus specific pathogenecity in several cell lines inoculated with the viral antigen and the tissue distribution of viral antigens to clarify the viral pathogenesis associated with acute hepatitis and haemorrhages in rabbit haemorrhagic disease by immunocytochemistry.

Materials and Methods

Cell culture : Several cell lines listed in Table 1 were used to propagate RHD virus. The media including EMEM(Gibco 410−1100), DMEM(Gibco 430−1600), and RPMI 1640(Gibco 430 − 1800) were used for cell culture as recommended by data sheet from the supplier. They were supplemented with 10% heat inactivated fetal bovine serum(FBS), amphotericin B(25 ㎛/㎖), and gentamycin(40 ㎍/㎖). The cultures were incubated in a humidified CO_2 incubator(5% CO_2/95% air)at 37℃.

Table 1. Cell lines and origin of cells

Cell line	Origin of cells
Vero	Kidney, African green monkey(Korea NIH)
HeLa	Epitheloid carcinoma, human cervix(Korea NIH)
MDCK	Kidney, dog(Korea NIH)
BHK21	Kidney, hamster(Suny at Stony Brook, USA)
H 9	T cells, human(NCI, NIH, USA)
HEL 299	Lung, human embryo(ATCC, USA)
WI 38	Lung, diploid human(ATCC, USA)
FRhK 4	Kidney, fetal rheusus monkey(ATCC, USA)
MOLT 3	Acute lymphoblastic leukemia(NCI, USA)

Exposure of RHD virus (RHDV) on cultured cells : Viral antigens were purified according to the method described previously.[8] Cultures of several cell lines were washed twice with calcium, magnesium free phosphate buffered saline solution and inoculated with 0.3㎖ of undiluted or 10% diluted purified virus. The haemagglutination activity titers of purified virus reached above 2048. After absorption for 45 minutes, the cells were then riped with maintenance medium and incubated at 34℃ in a CO_2 incubator. For serial passage of cells from flasks, cells were harvested and pelleted by low speed centrifugation at 257×g for 15 minutes at room temperature and resuspended in maintenance medium. The cells were riped weekly, and harvested at one week intervals. Uninoculated cell cultures for control were prepared and handled as above.

Preparation of hyperimmune serum : RHDV hyperimmune serum was prepared in New Zealand white rabbits. In brief, purified viral antigens were inactivated with 0.5% formalin, heated at 56℃ for 30 minutes, and emulsified 1 : 1 with complete Freund's adjuvant by mixing through two hypodermic syringes connected by a right angle stopcock. Two rabbits were injected intramuscularly at several sites with a total of 2㎖ of the viral antigens. After 2 weeks, the rabbits received a booster shot of viral antigen mixed in Freund's incomplete adjuvants. Thereafter identical boosters were given biweekly. The presence of antibody was determined by HI test and ELISA.[11] Nine weeks after the initial immunization, 30㎖ of blood was collected from the immunized rabbits. The blood was allowed to clot at 4℃ overnight and then centrifuged for 30 minutes at 4,000×g at 4℃ to clarify the sera. The sera were stored at -70℃ and used for immunohistochemistry.

Tissue processing : Twenty white rabbits from 4 to 6 month-old were intramuscularly inoculated with 0.2㎖ of liver suspensions of rabbits with haemorrhagic disease as used previously.[1] They died within 96 hours after inoculation, and were necropsied immediately after death. Tissues including liver, spleen, lungs, kidneys and brain were fixed in 10% buffered formalin, dehydrated and embedded in paraffin, and in part embedded in OCT compound for frozen sections. Sections with 5~7 ㎛ thickness were used for immunohistochemistry.

Immunohistochemistry : Immunohistochemical detection of viral antigens were done by indirect immunofluorescence and Avidin-biotin peroxidase complex (ABC) methods(Vector Kit).

For indirect immunofluorescence method, tissues fixed in cold acetone/methanol (1 : 1) were incubated with

10% normal goat serum for 45 minutes, labelled with anti-RHDV hyperimmune sera(1 : 100), and incubated with FITC conjugated goat anti-rabbit IgG(30 $\mu g/m\ell$: Sigma). At each step, tissues were washed with 0.05M PBS. After then examined under the fluorescence microscope(Nikon Diaphot-Polyva). Liver was chosen for a further study by ABC immunohistochemistry because liver showed strong positivity by indirect immunofluorescence technique.

For the ABC method, sections from the liver tissues were stained by the indirect immunoperoxidase method.[12] Endogenous peroxidase of the liver tissues were blocked with 0.05% H_2O_2 in methanol, washed with PBS, reacted with 10% normal goat serum, and incubated with anti-RHDV(1 : 100~500) sera. After washing with buffer, sections were treated with biotinylated anti-Rabbit IgG, labelled with peroxidase conjugated ExtrAvidin (Sigma) or Avidin(Vector) as recommanded by manufacturer. DAB(3, 3'-diaminobenzidine Tetrahydrochloride) and AEC(3-amino-9-ethylcarbazole) were used as a substrate as shown previously.[12]

For the control, primary antiserum was omitted on the adjacent sections.

Results

Tissue distribution of viral antigens in rabbits with RHD was studied by immunofluorescence and Avidin-biotin complex methods.

Susceptibility of RHD virus on cultured cell lines : Various cell lines were inoculated with RHD virus and examined by HA test. None of the cultures tested showed virus-induced characteristics such as CPE. No significant HA titer was recognized in culture fluid and cell lysates exposed with RHD virus.

Immunofluorescence : Sepcific fluorescence was observed in the liver, spleen, and kidneys. There was no obvious immunofluorescence in the brain and lungs of RHD. Viral antigens were found in the liver from all the infected rabbits, but in some cases there was also positive staining in the spleen, and kidneys.

In the liver, strong immunoreactivity was found in the hepatocytes of periportal triad lesions and peripheral lesions of the hepatic lobules, where immunoreactive product was shown in the form of granules and clumps in the cytoplasm of hepatocytes(Fig 1). Immunoreactivity appeared in the cytoplasm of macrophages in the red pulp of the spleen(Fig 2) and in the renal mesangial cells and endothelial cells of the glomeruli(Fig 3).

ABC methods on the liver : The hepatic tissue from all the infected rabbits showed strong immunoreactivity. The immunoreactive products by ABC method were more suitable for visual evaluation than immunofluorescence. The intensive staining of the cytoplasm of infected hepatocytes and sinusoidal cells allowed the exact distinction from adjacent uninfected cells(Fig 4). In all the rabbits examined, the causative antigen was detected in degenerative and necrotic hepatocytes of the liver tissues, and the area involved coincided with histopathological lesion on the serial liver sections. The immunoreactive pattern of viral antigens in the hepatocytes was a fine granular type, and the distribution pattern of immunoreactive products was diffusely scattered in the cytoplasm or marginated at the periphery of the cells (Fig 5). The immunoreactive products were better in the sections by the ExtrAvidin peroxidase staining method than that of Avidin peroxidase staining method (Fig 6).

Discussion

RHD is an acute infectious disease characterized by sudden death, hepatitis and hemorrhages only in rabbits. Tissue distribution of causative virus in RHD was studied by immunofluorescence and ABC methods in this study. RHDV antigens were mainly present in the liver, spleen and kidneys. It suggests that RHDV has a tropism of hepatic cells after infection, and has an intimate relationship with mononuclear phagocytic system cells in the liver and spleen after infection. However, there were some difference on the distribution of RHDV antigens by the techniques and authors.

Weiping et al[13] confirmed that RHDV antigens have been found in the liver, spleen, kidneys, lungs and cerebrum at early stage of infection by HA test and immunohistochemistry. This minor discrepancy might be caused by the detecting method, duration of disease, age of rabbits, and route of infection and some other factors.

Concerning the viral tropism in RHD studied by Hao et al[14], RHDV was proved to a pantropic virus injuring most tissues and cells. Of these tissues, the principal target cells were hepatocytes and endothelial cells.

RHDV also has a tropism of vascular endothelial cells associated with disseminated intravascular coagulation (DIC)[15] and haemorrhages in many organs.[14]

RHDV has been classified in a calicivirus by many workers.[4,5,8] Caliciviruses replicate in the cytoplasm of the infected cells *in vivo* and *in vitro*.[16] On the distribution of viral antigen in infected cells, either intranuclear or intracytoplasmic viral antigens by EM and immunohistochemistry were found.[14] An immunoreactive product in this study was mainly found in the cytoplasm of hepatocytes and a few sinusoidal cells. This suggests that RHDV in RHD replicate in the cytoplasm of hepatocytes leading to acute hepatic dysfunction and circulatory disturbance. The discrepancy between two locations remained unsolved until now.

For the diagnosis of RHD, ABC and immunofluorescence methods might be useful in natural and experimental infections, and the liver has been supposed to be a good organ for the study of RHD.

Summary

Tissue distribution of RHDV in rabbits were examined by immunofluorescence and ABC methods. Tissues including liver, spleen, kidneys, lungs and brain were frozen, cut in a crycut, and fixed in 10% buffered formalin, embedded in paraplast, and cut 5~7 μm thickness. Sections were immunostained with primary antiserum and conjugated second antibodies as recommended by manufacturer.

None of the cultures tested showed virus-induced phenomena.

Immunoreactive products were commonly found in the liver, in some cases there were also positive staining in the spleen and kidneys. Other organs showed weak or insignificant immunoreactions. By ABC method on the formalin-fixed, paraffin-embedded liver tissues, strong immunoreactivity was found in the periportal triad lesions and peripheral lesions of the hepatic lobules. Immunoreactive products showed diffuse fine granular in the cytoplasm of hepatocytes and sinusoidal cells. In some cells, immunoproducts marginate at the periphery of the cells. The intensive staining of the cytoplasm of infected cells allowed their exact differentiation from surrounding uninfected cells. The positive area involved coincided with histopathological lesion on serial liver sections.

In conclusion, liver was proved to be a consistent target organ in RHD, and the immunoperoxidase method in the section of formalin-fixed, paraffin-embedded hepatic tissue could be broadly used for the routine diagnosis of the disease.

Legends for figures

Fig 1. Liver stained by indirect immunofluorescence test with FITC-conjugated goat anti rabbit IgG. A wide spectrum of fluorescence is observed in hepatocytes. × 400.

Fig 2. Spleen stained by indirect immunofluorescence test with FITC-conjugated goat anti rabbit IgG. Specific fluorescence is evident in the macrophages of red pulp. × 400.

Fig 3. Kidney stained by indirect immunofluorescence test with FITC-conjugated goat anti rabbit IgG. Specific fluorescence is observed in the glomeruli. × 400.

Fig 4. Immunohistochemical staining by using ABC kit in a section of formalin-fixed, paraffin-embedded hepatic tissue. Infected cells are readily identifiable from uninfected cells by intense brown staining (seen here as black) in their cytoplasm. × 134.

Fig 5. Immunohistochemical staining by using ABC kit in a section of formalin-fixed, paraffin-embedded hepatic tissue. The dense granules are visible in the cytoplasm of hepatocyte. × 400.

Fig 6. Immunohistochemical staining by using ExtrAvidin Biotin Staining kit in a section of formalin-fixed, paraffin-embedded hepatic tissue. The infected cells with intense red staining (seen here as black) in the cytoplasm of hepatocytes are readily identifiable. Hematoxylin counterstain, × 400.


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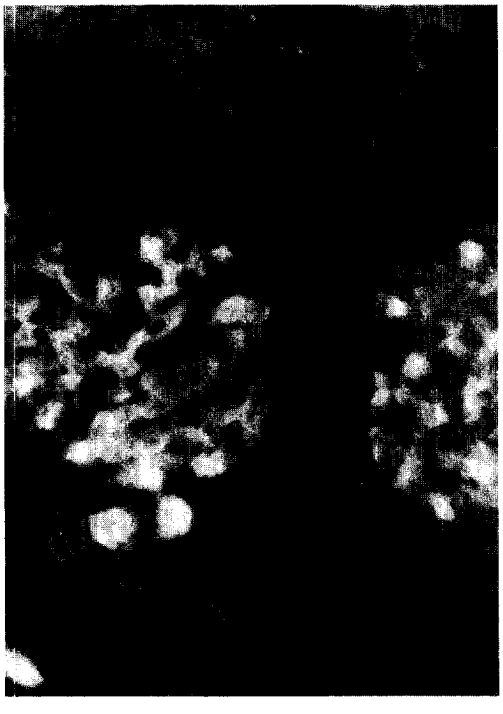

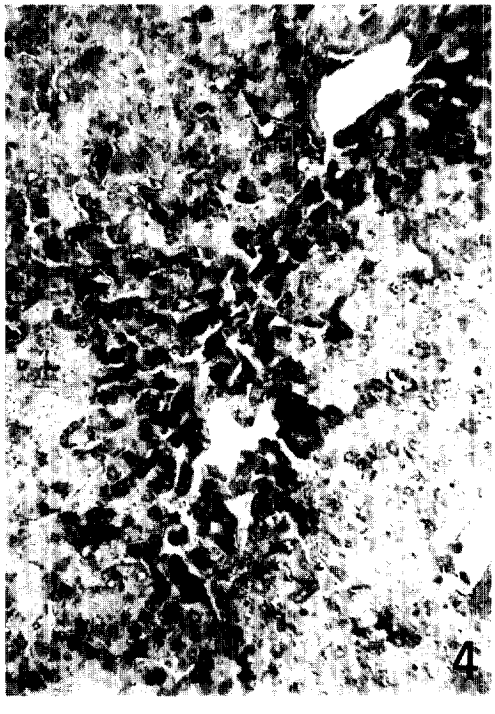
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