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Further characterization of the causative virus of rabbit viral hepatitis, so-called rabbit haemorrhagic disease in Korea

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국내에서 발생한 토끼 바이러스성 간염 소위 토끼 출혈병 바이러스의 성상

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초 록 : 국내에서 발생한 토끼 바이러스성 간염 소위 토끼 출혈병의 원인 바이러스를 감염토끼의 간조직으로 부터 분리정제한 후 바이러스의 핵산과 구성 단백질의 특징을 관찰하였던 바 다음과 같은 결과를 얻었다.

토끼간염바이러스는 분자량이 약 54 kilodalton인 한개의 구조단백을 가진 RNA 바이러스이며 바이러스 핵산의 크기는 약 7.5 kilobases로 나타났고 바이러스의 RNA는 배양세포에서는 감염을 일으키지 않았다.

바이러스 구성단백의 양상과 핵산의 크기 등을 종합해 볼 때 토끼의 간염 바이러스는 Caliciviridae에 속하는 것으로 간주된다.

Key words : rabbit, hepatitis, calicivirus, viral protein, viral RNA.

Introduction

A new viral disease of rabbits has been described in China[1,2] and in Korea[3~6] since 1984. The disease is characterized by high morbidity and mortality only in rabbits. The conspicous pathologic findings were hemorrhages in the lungs, liver, kidneys and heart of naturally infected rabbits. In experimentally infected rabbits, marked lesions were found in the liver.

Rabbit hemorrhagic diseases have been also reported in Europe[7,8] and in south America.[9] The agent has been classified among picornaviruses[2,4,5], caliciviruses[7,8] and parvoviruses.[9]

The nature of the causative agent of this disease is not well characterized because the virus couldn't be cultivated in established cell lines[4], although three groups of these viruses above have their own characteristics. Caliciviruses has a single major polypeptide and some minor

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proteins, which was once classified into the subgroup of the four major structural protein-containing picornaviruses.[10~14]

In an effort to further study rabbit hepatitis virus, we have purified this virus from the liver of infected rabbits and further characterized the structural proteins of this virus and viral RNA.

Materials and Methods

Purification of the virus : Viruses were purified according to the method described by Dobos et al[15] with slight modification. Liver homogenates(20% in PBS) of the experimentally infected rabbits was clarified twice for 20 minutes at 25,700 x g at 4℃ using JA-21 fixed angle rotor(Beckman, J2-21 M/E centrifuge). The supernatant was collected and pelleted on sucrose cushion followed by ultracentrifugation for 4 hours at 240,000 x g at 4℃ using SW 40 rotor(Beckman L8-80 M ultracentrifuge). The pellet was resuspended in 5mM TNE buffer(5mM Tris-HCl, 5mM EDTA, 100mM NaCl, 0.03% NaN3, 0.02% Tween-20, pH 7.2), and layered on the top of the continuous sucrose gradient 10-45%(wt/vol). Virus band was collected at the interface with a syringe after overnight ultracentrifugation at 160,000 x g at 4℃. After dialysis of purified viruses, haemagglutinating activity using human "O" type erythrocytes was checked and stored at -70℃ until used. The highest HA titer(above 4096) was found in fractions 26-27 in this experiment (Fig 1).

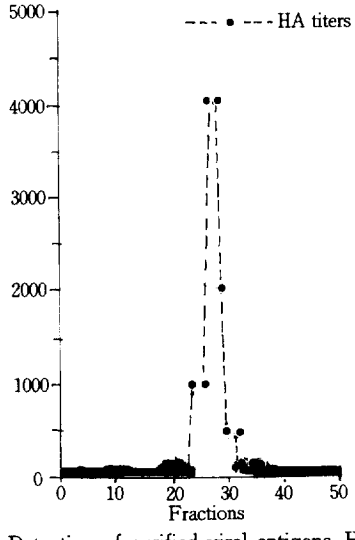

Fig 1. Detection of purified viral antigens. HA titers were reached above 4, 096.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) : Electrophoresis of purified virus was performed on 12.5% gel slabs by the methods of Laemmli[16], using marker proteins ranging from 30,000 to 94,000 daltons. The virus and standard proteins were boiled for 2 minutes in sample buffer(1% SDS, 0.1% 2-mercaptoethanol, 0.05% bromphenol blue, and 5% glycerol) and layered on the top of gels. After electrophoresis, the gels were stained with coomassie blue.

Isolation of viral nucleic acid[15,17] : Viral nucleic acids were precipitated with an equal volume of phenol and twice with chloroform/isoamylalcohol(24 : 1). After ethanol precipitation in the presence of 0.15 M NaCl, the pellet was washed with 70% ethanol, dried and resuspended in distilled water. The viral genome was identified through electrophoresis on 0.8% agarose gel containing 50 μg /mℓ ethidium bromide.

Transfection of viral nucleic acids : Three types of cell lines were used for transfection. Monolayers of Vero, HeLa and HEL 299 cells grown in EMEM supplemented with 10% FBS were used for transfection experiments. Transfections were carried out essentially as described by the Koch method.[18] Extracted RNA(0.8mg /mℓ) was diluted in distilled water mixed with an equal volume of 1mg/mℓ of Dextran (Pharmacia) dissolved in 2X Hepes buffer(275 mM NaCl, 10mM KCl, 42 mM Hepes, 12 mM glucose, pH 7.8) and placed for 30 minutes at 0℃. Recipient monolayer cells were washed once with PBS and then incubated for 30 minutes at room temperature with 0.2mℓ of RNA-DEAE-Dextran mixture on the six well plates. Plaques were allowed to develop by incubating the plates at 37℃ in EME medium containing 5% FBS or under semisolid solution(1.6% Gum tragacanth) containing the same medium.

Yeast RNA(Gibco) was used as a negative control and virion RNA of poliovirus type 1 strain Mahoney(PV 1(M)) was used for positive control(kindly provided by Dr. CK Lee, KRICT).

Results

Electrophoretic protein patterns of the virus : The protein structure of rabbit hepatitis virus revealed a single major protein in 12.5% gels stained with coomassie blue(Fig 2). The single band of viral protein was identified between 43 kilodalton (KD) and 67 KD marker pro-

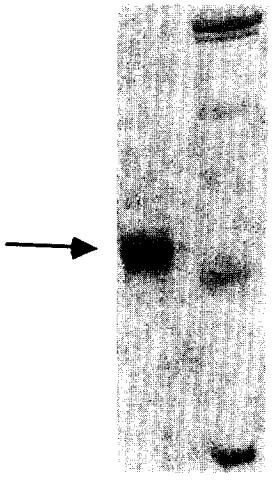

Fig 2. SDS-PAGE pattern of purified rabbit hepatitis virus(lane 1) and molecular size markers(lane 2;94, 67, 43 and 30KD). An Arrow shows the position of the one major viral protein.

teins. The estimated molecular weight of this virus is about 54 KD.

Isolation of viral nucleic acids : Viral nucleic acids were released from purified viruses by phenol and chloroform extraction, and were electrophoresed 0.8% agarose gel with TAE buffer(Fig 3). The viral nucleic acid migrated close to 7.5 kilobases of the poliovirus RNA(poliovirus type 1 strain Mahoney (PV 1 (M)).[17]

Transfection of viral nucleic acids : Virion RNA of PV1(M) developed 70 PFU per 1 ㎎ RNA into HeLa, HEL 299, vero cell lines. However nucleic acids of rabbit hepatitis virus showed no significant virus-specific pathogenecity *in vitro* system.

Discussion

Outbreaks of a new viral disease in rabbits have been reported since 1984 in many countries.[1~9] The nature of the causative agent of this disease has been disputed among picornaviruses[3~5], caliciviruses[7,8] and parvoviruses.[9]

Caliciviruses are small RNA viruses which are unique among picornavirus, except that the structural proteins of caliciviruses are different from those of picornaviruses.[10] A single major capsid protein differentiates the caliciviruses from picornaviruses, which have 4 structural proteins. A new virus causing rabbit sudden death initially fulfilled the physicochemical criteria of picornaviruses.[4]

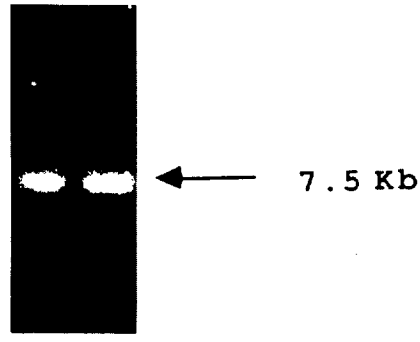

Fig 3. Electrophoretic separation of viral nucleic acid of rabbit hepatitis virus. Lane 1 : viral RNA (2 ㎍). Lane 2 : PV 1(M) RNA(4 ㎍).

The protein structures of rabbit hepatitis virus in this experiment suggest that Korean isolate of this virus is a candidate calicivirus. The molecular weight of candidate caliciviruses varied depending on animal species.[13] Canine caliciviruses possess a single major band of capsid protein of 58,000 daltons which was slightly less than those of other caliciviruses.[12,13] In this study, a single major protein of rabbit hepatitis virus was calculated to be about 54,000 daltons similar to the data described by Wu et al.[19], which is slightly less than 60,000 daltons of European isolates of rabbit caliciviruses.[7,8] The slight differences of viral proteins between two groups may be caused by techniques according to authors.

The viral nucleic acid of rabbit hepatitis virus in this study was about 7.5 kb close to the poliovirus RNA. Recently, the size of viral RNA of rabbit haemorrhagic disease virus was calculated to be about 8 kilobases.[7] In addition, isolated nucleic acid was treated with RNAse A(Sigma) and DNAse I (Sigma). This viral RNA was found to be resistant to DNAse I, contrary to the findings treated with RNAse A(Data not shown). Inactivation of rabbit hepatitis virus by RNAse A digestion means that this virus belongs to RNA virus. Consequently DNA viruses including parvoviruses may be excluded.

Further study will be directed toward the differences of viral proteins and viral RNA. Denaturing gel system with RNA size markers will determine the accurate size of the rabbit hepatitis virus nucleic acid.

These results show that a calicivirus is the causative agent of rabbit hepatitis, so-called rabbit haemorrhagic disease outbroken in Korean. The further characterization and its relatedness with other caliciviruses are in progress.

Summary

The causative virus causing rabbit hepatitis has been further characterized by evaluating viral proteins and viral nucleic acids of purified viruses from the liver of the experimentally infected rabbits.

Rabbit hepatitis virus has one major structural protein of 54 kilodaltons and some minor proteins. Vrial RNA was resistant to DNAse Ⅰ. The size of viral nucleic acid of this virus was calculated to be about 7.5 kilobases.

These findings indicate that rabbit hepatitis virus belongs to the family Caliciviridae.

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