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Natural Infection of Nude Mice with Low-virulent Mouse Coronavirus

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With 2 figures and 5 tables

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Summary

The outbreak of wasting disease of nude mice occurred in the laboratory colony of a Pharmaceutical Company. The viruses producing cytopathic effect with syncytium formation were isolated from the wasted nude mice by DBT cells, and were identified as mouse coronavirus by direct immunofluorescence. The nude mouse colony was closed and all the nude mice (about 500) were killed by the reason of disease control. At autopsy about 60 % of nude mice showed necrotic hepatitis. By the virus isolation to see the source of contamination, viruses were isolated from the feces of apparently healthy mice of ICR, CDF1, DBA/2 and C3H, and from human cancer cell line stocked in liquid nitrogen. In experimental infection, the isolates produced only mild hepatitis in ICR mice treated with cortisone. By cross-neutralization test, the nude isolate reacted closely with the virus from C3H mice but not with the virus from cancer cell line. The isolates from nude and C3H mice produced experimentally wasting disease with necrotic hepatitis in nude mice. These findings suggest that wasting disease in nude mice might be caused by low-virulent mouse coronavirus shed in feces from C3H mice introduced before the outbreak of disease.

Introduction

Athymic and immunodeficient nude mice have been widely used for immunologic as well as cancer researches. The mutant mice are highly susceptible for common infectious agents such as Sendai virus and mouse coronavirus (murine hepatitis virus; MHV), suffering from "wasting disease" characterized by depression and body weight loss (1, 3, 11, 16, 18–20).

PANTELOURIS (15) described that wasted and dead nude mice frequently had multiple necrotized foci in the liver. From wasted nude mice with hepatitis, SEBESTENY and HILL (16) isolated a low-virulent MHV, and many authors isolated low-virulent MHV strains (1, 11, 12, 13, 19). Mouse coronavirus has been recognized as a major pathogen causing wasting syndrome in nude mice (3, 17).

During November and December 1986, acute deaths occurred in nude mice at a laboratory colony in Pharmaceutical Company near Tokyo. At autopsy necrotized lesions were detected in the liver of some wasted and dead nude mice. This report deals with isolation of a low-virulent MHV from the cases and epizootiology.

Material and Methods

Animal room: Three hundreds to 600 athymic nude mice aged 6- to 15-week old had been usually kept for using various studies, since 1983, and about 200 nude mice were monthly introduced from a commercial breeder (Japan SLC, Hamamatsu). Ten mice each were kept in plastic cages (215×320×130 mm) with a filter cap within a unit with HEPA-filter. They were given gamma irradiated commercial pellet (Clea Japan) and filtrated aseptic water by automatic system. Animal rooms were supplied with filtrated fresh air. In respected rooms near to this colony (room 8), inbred and hybrid mice of various strains were kept, as illustrated in Fig. 1. The nude mouse colony was closed in late December 1986 and about 300 of 500 nude mice were revealed to have liver necrosis at autopsy.

Immunofluorescence and virus isolation: Direct immunofluorescence was performed on frozen sections of the liver by using anti-MHV-2 rabbit antibody conjugated with fluorescein isothiocyanate (4—5). Ten % homogenates of liver and spleen tissues were prepared in Eagle's minimum essential (MEM) and after centrifugation at 3,000 rpm for 10 min, the supernatant was inoculated into DBT cell cultures (4, 5). For isolation from feces, a spoonful of freshly collected feces was homogenized in 20 ml of MEM containing penicillin G (2,000 IU/ml) and streptomycin (2,000 μg/ml) and the supernatant was inoculated into DBT cell cultures after centrifugation at 3,000 rpm for 10 min. The DBT cells were grown at 37 °C in MEM containing 10 % newborn calf serum (NCS), 10 % tryptose phosphate broth (TPB) and kanamycin (0.06 mg/ml). For maintaining the cells and harvesting the virus, NCS concentration was reduced to 5 %. Infected DBT cells grown on coverslips were fixed with cold acetone for 10 min, and the coverslips were subjected to immunofluorescence (4—5).

Plaque assay: Isolates were assayed for infectivity by plaque method described previously on DBT cell system (4, 5). As reference viruses, MHV-2 (14), MHV-NuA (11), feline herpesvirus (FHV) strain 406 and feline calicivirus (FCV) strain 410 (10) were used. FHV and FCV were also assayed by plaque method on CatS+L−cells (10). DBT and CatS+L−cell cultures prepared in 60 mm plastic dishes (Terumo, Japan) were washed once with MEM and inoculated with 0.2 ml of virus material. After virus adsorption at 37 °C for 90 min, the cultures were overlaid with 5 ml of agar medium consisting of maintenance medium and 1 % Bacto agar (Difco) and then incubated at 37 °C for 48 h in 5 % CO_2 incubator. For plaque count the cell cultures were overlaid with agar medium containing 1 : 10,000 neutral red. The infectivity was expressed by plaque-forming units (PFU).

Physicochemical properties of the isolates: Effect of 5-iodo-2-deoxyuridine (IUDR) on virus growth, sensitivity to ether and chloroform, pH 3.0 sensitivity, sensitivity to sodium deoxycholate (SDC) and filtration test were examined as described previously (6).

Pathogenicity of isolates for mice: Four-week-old male ICR mice as well as 8-week-old male athymic nude mice were obtained from commercial breeding colony (Japan SLC, Hamamatsu), which

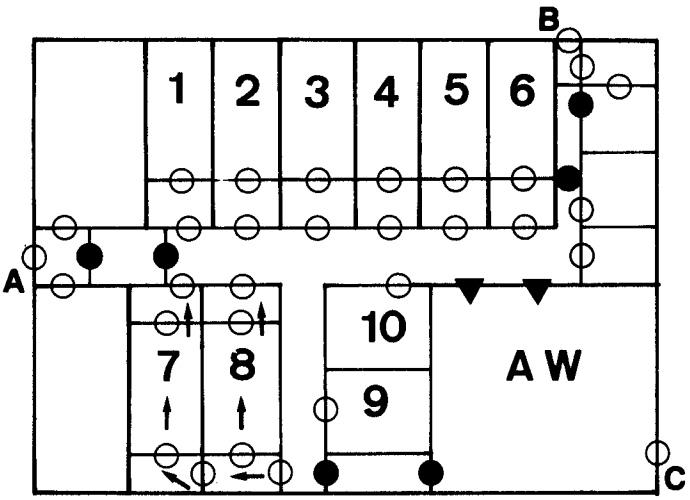

Fig. 1. Arrangement of animal rooms. The symbols and abbreviations indicate as follows. 1—7, 9, 10: rooms for rats and mice, 8: room for nude mice, AW: autoclave and wash room, ○ ●: doors, ● ●: air shower room, ▼: autoclave, ← : one way

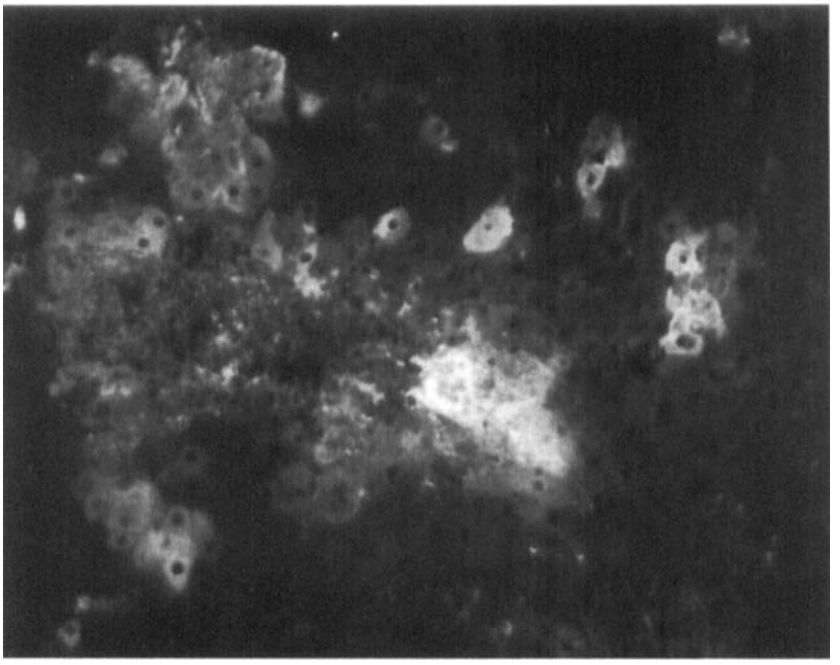

Fig. 2. Viral specific antigen in the cytoplasm of hepatocytes surrounding necrotic foci by direct immunofluorescence using anti-MHV-2 rabbit antibody

had been checked for being free from MHV infection. They were kept in vinyl isolators and given autoclaved commercial pellets and water. Mice were inoculated with the virus by intranasal (i.n.) (0.02 ml) or intraperitoneal (i.p.) (0.2 ml) route. Hydrocortisone acetate (2.5 mg) was subcutaneously administrated shortly before virus inoculation.

Immunological study: For preparing antiserum, ICR mice were inoculated i.p. with each isolate 3 times at 1 week intervals and were sacrificed for collecting the blood 2 weeks after the last injection. For neutralization test (NT), 1 ml of serial dilutions of antiserum in MEM were mixed with an equal volume of virus material (200 PFU / 0.2 ml). The mixtures were incubated at room temperature for 30 min, and then inoculated into DBT cell cultures. The number of plaques was counted as described above, and neutralizing antibody titers were expressed by 50 % plaque reduction.

Results

The immunofluorescence with the frozen sections of the nude mouse liver showing focal necrosis, which were sampled in December 1986, revealed specific antigen within the cytoplasm of hepatocytes surrounding necrotic foci (Fig. 2). Within 24 h after inoculation of 12 liver and 2 spleen samples onto DBT cells, were isolated 6 agents showing cytopathic effect (CPE) characterized by syncytium formation (Table 1). All of the 4 liver samples

Table 1. Virus isolation from wasted nude mice on DBT cells

Organ	Necrosis*	Positive/Tested	Positive case (Code)
Liver	+	4/4	IN-3, -4, -5, -12**
	−	0/8	
Spleen	−	2/2	IN-4(S), -12(S)

* Macroscopically focal necrosis. ** Positive for MHV antigen by direct immunofluorescence.

Table 2. Virus isolation from feces of mice on DBT cells

Room*	Mouse strain	Age in weeks	Positive/tested
1	CDF1, DBF1	6–7	0/10
2	BDF1, CDF1	9–16	0/10
4	BDF1, DBF1	6–10	0/10
5	CDF1, MRL/1, NOD	8–16	0/6
6	BALB/c, C3H	?–22	1**/2
7	C57BL, ICR, CDF1, DBA/2	7–9	3***/6
9	CDF1	7	0/4
10	BALB/c	9	0/4

* See Fig. 1; ** C3H; *** ICR, CDF1, DBA/2.

with focal necrosis were positive for isolation. On coverslip cultures of DBT cells inoculated with 6 isolates, MHV-specific antigen was detected within the cytoplasm of syncytial DBT cells.

After closing the infected nude mouse colony in late December 1986, 52 fecal specimens were collected from apparently healthy mice of the neighboring animal rooms and they were examined for virus isolation. As shown in Table 2, 4 cytopathic agents were isolated from the fecal samples of ICR, CDF1 and DBA/2 mice reared at room 7 and C3H mice at room 6. Among these strains, other strains of mice than C3H were introduced in December 1986 and immunosuppressed for tumor transplantation. In February 1987, the virus isolation was again made, revealing that the feces of C3H mice at room 6 were again positive. C3H mice were introduced in June 1986 from a private institute.

Attempts were made to isolate the virus from transplantable tumor cells stocked in liquid nitrogen, as shown in Table 3. Among 15 cell lines examined, only one isolate (designated as Sq) was detected from human squamous cell carcinoma, Sq/S cell line, which had not been transplanted into nude mice since March 1980. All the isolates produced in 48 h small clear plaques measured 0.8 to 1.0 mm in diameter on DBT cell cultures.

Physicochemical properties of the isolates: The growth of 8 isolates form nude mice, C3H mice and tumor cells on DBT cell cultures was not affected with IUDR, but completely inactivated by ether and chloroform treatments. They were resistant to pH 3.0. After treating with 0.1 % SDC, the isolates were completely inactivated while 0.01 % SDC was ineffective. After heating at 50 °C, virus titers decreased to 1 : 1,000 for 30 min and complete inactivation was seen after 60 min. These isolates were passed through 200 and 100 nm membrane filters (Sartorius, West Germany) but not 50 nm ones. As control viruses, FCV and FHV behaved as expected.

Table 3. Pathogenicity of the isolates for 4-week-old ICR mice by i. p. route

Virus	Inoculum (PFU)	Cortisone*	Dead/Tested	Liver lesion			
				+++	++	+	–
IN-12	8×10^4	+	0/5		1	4	
	8×10^4	–	0/5				5
F-2D	1×10^4	+	0/5			4	1
	1×10^4	–	0/5				5
Sq	2×10^4	+	0/5	1	4		
	2×10^4	–	0/5				5
NuA	2×10^4	+	5/5 (5.2)**	5			
	2×10^4	–	3/5 (6.3)	5			
MHV-2	6×10^2	–	5/5 (2.4)	5			

* 2.5 mg administered subcutaneously; ** mean time to death in days.

Table 4. Cross-neutralization test between IN-12, F-12D, NuA and MHV-2

Virus	(Source)	Antiserum to				
		IN-12	F-2D	Sq	NuA	MHV-2
IN-12	(Nude liver)	1 : 1,280*	1 : 1,280	1 : 80	1 : 20 >	1 : 20 >
F-2D	(C3H feces)	1 : 1,280	1 : 2,560	1 : 40	1 : 20 >	1 : 20 >
Sq	(human carcinoma)	1 : 20 >	1 : 20 >	1 : 1,280	1 : 20 >	1 : 20 >
NuA		1 : 20 >	1 : 20 >	1 : 80	1 : 640	1 : 20 >
MHV-2		1 : 80	1 : 80	1 : 80	1 : 20	1 : 1,280

* Expressed by 50 % plaque reduction.

Pathogenicity of the isolates for mice: As shown in Table 4, all the mice inoculated i. p. with IN-12, F-2D and Sq isolates survived for 14 days postinoculation (p. i.) even with cortisone treatment. At autopsy they showed mild liver lesions whereas all the mice inoculated with reference viruses, MHV-2 and NuA, died of acute hepatitis within 7 days p. i. The Sq virus was more virulent than IN-12 and F-2D in producing the liver lesion.

As shown in Table 5, antisera to IN-12 and F-2D were shown to react with both IN-12 and F-2D but they did not react with Sq. IN-12 and F-2D also did not react with antisera to MHV-2 and NuA. These experiments suggest that IN-12 is antigenitically similar to F-2D.

Three nude mice were inoculated i. n. with 1.3×10^4 PFU of IN-12 or with 2.5×10^3 PFU of F-2D. After inoculation the IN-12 infected mice died at 17, 18 and 20 days p. i. showing wasting syndrome. The mice infected with F-2D also died at 24, 28 and 41 days p. i. At necropsy all the dead mice showed the severe necrotized liver, being similar to natural nude cases. The virus was recovered from all the affected livers.

Discussion

An outbreak of wasting disease in nude mice was revealed to be due to low-virulent MHV infection. HIRANO et al. (7) and HIRASAWA et al. (12) isolated low-virulent MHV from feces of apparently healthy mice reared in infected mouse colony. FOX et al. (2) and MARUO et al. (13) described infection of MHV in mouse ascitic myeloma and human cells transplanted into nude mice, respectively.

In this study, MHV was isolated from the wasted nude mice. By the attempts to isolate the causative agent, MHV was isolated from feces of apparently healthy mice and from transplanted human cancer cells to nude mice. While MHV was isolated from the human squamous cell carcinoma stocked in liquid nitrogen, little possibility was considered to cause MHV infection in nude mice by Sq isolate because the cell line was not transplanted since March 1980. The source of MHV infection might be from feces of ICR, CDF1, DBA/2 and C3H mice reared at the neighbor of infected nude mice. However, the strains of mice other than C3H were introduced in December 1986 when the outbreak of wasting disease in nude mice was already wide-spread. Probably C3H mice had been

Table 5. Pathogenicity of the isolates for 8-week-old athymic nude mice by i. n. route

Virus	Inoculum (PFU)	Hepatitis	Mortality	Mean time to death
IN-12	1.3×10^4	3/3*	3/3**	18.9 (17—20)***
F-2D	2.5×10^3	3/3	3/3	31.0 (24—41)

* Positive/Tested; ** Dead/Tested; *** Mean time to death in days with range in parenthesis.

inapparently infected with MHV when they were introduced into this laboratory from a private institute without checking for MHV infection in June 1986. C3H mice had been kept for breeding since June 1986, and they might be responsible for the MHV infection in nude mice. The authors previously reported the isolation of a low-virulent MHV from persistently infected mouse colony, and suggested that MHV might be transmitted among the infant and young mice (7). On the other hand, MHV isolation from human cell line strongly suggests the presence of MHV before March 1980 when this cell line was stocked in liquid nitrogen.

Physicochemical properties of those isolate were similar to those of MHV-2 (6) and other strains (9). The nude isolate IN-12 as well as F-2D isolate from C3H mice were of lower virulence for ICR mice than the Sq isolate from Sq/S cells. By the cross-NT, the isolates, IN-12 and F-2D shared common antigens. However, both IN-12 and F-12D viruses can produce the wasting disease in nude mice with necrotic hepatitis after i. n. inoculation. From these data obtained, sudden death of nude mice was due to the infection of low-virulent MHV, which has been shed from C3H mice.

Although such type of low-virulent MHV are able to produce hepatitis in euthyic mice only when treated with immunosuppressant (3, 8), persistent infection can be established in nude mice resulting in wasting disease (3, 18). The wasting disease of nude mice due to MHV infection is a serious problem in cancer and immunological studies using nude mice. The control for low-virulent MHV infection in nude mice can be made by viral isolation from feces of apparently healthy mice as well as nude mice.

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