Characterization of Hog Cholera Virus

I. Determination of Buoyant Density

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Received for publication 21 September 1966

ABSTRACT

HORZINEK, MARIAN (Tierärztliche Hochschule, Hannover, West Germany). Characterization of hog cholera virus. I. Determination of buoyant density. *J. Bacteriol.* 92:1723-1726. 1966.—Hog cholera virus was subjected to cesium chloride density gradient centrifugation. Most of the infectious activity was detected in fractions with densities between 1.15 and 1.20 g/ml, with a peak at 1.16 g/ml. Infectivity was assayed by use of either the exaltation of Newcastle disease virus method or the hemagglutination exaltation and inhibition of cytopathic effect method.

Little is known about the chemical and physical properties of hog cholera virus (HCV). There is only evidence that HCV is a chloroform-labile, trypsin-sensitive ribonucleic acid virus (5, 14). Its size was determined to be in the range of 15 to 44 $m\mu$ (5). These scant data do not allow one to decide whether HCV belongs to one of the known animal virus groups, or whether it possesses a unique structure. Therefore, the suggestions made by some authors about the classification of HCV are rather speculative. Dinter (5) considers HCV to be related to group B arboviruses; others (4, 6, 18) have shown an antigenic relationship between HCV and bovine viral diarrhea virus. Bovine viral diarrhea virus has been classified as a member of the myxovirus group (6, 7), but these results also have been questioned (27). Wilner (29) thus listed the agent under "possible myxoviruses."

The present report, the first in a series of studies dealing with the properties of HCV, presents results of density gradient centrifugations performed to concentrate and purify the virion for further analysis.

MATERIALS AND METHODS

Virus. The following strains of HCV were used in this study: Behring, a freeze-dried serum taken from pigs during the viremic phase (lot 8721); München 1, blood taken from pigs during the viremic phase; ALD, lyophilized infectious tissue culture material (40th passage in pig kidney cells). All these strains are virulent for pigs.

The Behring strain was used either reconstituted or after one passage in pig kidney (PK) cells. The München 1 strain had undergone two or three passages in

swine testicle (ST) cells. The ALD strain was propagated in PK cells in our laboratory; infectious tissue culture media of the third to the sixth passage were chosen.

Monolayer cultures of PK and ST in 1,000-ml prescription bottles were inoculated with 10^5 ID₅₀ of HCV. For harvesting of virus, culture media were collected 5 days after infection and clarified by low-speed centrifugation (30 min at 5,000 $\times$ g). The pooled supernatant liquids were used for density gradient centrifugation. All materials were stored at 4 C.

The hemagglutination exaltation and inhibition of cytopathic effect (HEIC) test was performed with the Sato strain of Newcastle disease virus (NDV) (21); the Miyadera strain of NDV was used in the exaltation of Newcastle disease virus (END) test (12, 13, 16). Both NDV strains were propagated in the allantoic cavity of the developing egg inoculated after 10 days of incubation; the fluid was harvested immediately after death of the embryo. The pooled allantoic fluids were assayed for infectivity (19) and stored at -70 C in 1-ml portions.

Sera. Hog cholera-hyperimmune serum (Italy serum) and serum of a colostrum-deprived piglet raised in isolation (normal serum) were heated for 30 min at 56 C and then used in the neutralization test (8).

Tissue culture media employed in the HEIC or END test were supplemented with calf serum; calf sera were obtained from the regional slaughterhouse and were separately tested for absence of inhibitory activity (12).

Tissue culture. We prepared PK cells in tubes as already described (8) and adjusted the packed cell volume to 2% of the final suspension.

We obtained ST cultures by the procedure of Kumagai et al. (13). The testicles, which had not always been treated aseptically after castration, were immersed in 96% ethyl alcohol. The alcohol was

ignited, and epiorchium and tunica albuginea testis could then be easily removed.

The medium used for propagation of HCV, for both PK and ST cells, consisted of Earle's balanced salt solution with 0.5% lactalbumin hydrolysate (Difco, no. 5996-01) and 10% inactivated calf serum.

Titration. For the HEIC test, we closely followed the procedure described by Nishimura (21), except for inoculations of HCV dilutions into tube cultures in 1-ml volumes. The culture medium was decanted 9 days after infection with HCV, and 2.5×10^6 plaque-forming units of NDV, suspended in 1 ml of Hanks' balanced salt solution with 0.5% lactalbumin hydrolysate, was pipetted into each tube. The amount of NDV-hemagglutinin was determined 2 days later for each tube by use of the microdilution technique of Sever (25).

For the END test, the original technique (12) was modified in that 1-day-old ST cultures had to be used for titration, because piglets were not regularly available for castration, and density gradient centrifugations had to be run for at least 15 hr. In our experiments, we made the same observation as Loan (15) that delay of the HCV inoculation for 1 day or more after seeding the cells does not adversely affect the titers. In most cases, they were higher than those obtained by the original procedure (12). We changed the medium on the 2nd and 5th day, and, on the 7th day after infection of the cultures with HCV, we inoculated 10^6 plaque-forming units per ml of NDV (strain Miyadera). All titrations were performed in 0.5-log steps with three tubes per dilution. Infectivity titers were calculated according to the formula of Kärber (11).

Plaque titers of both NDV strains were determined in chick embryo monolayer cultures in plastic petri dishes according to the technique of Mussgay and Rott (19).

Neutralization and chloroform-resistance tests were carried out as described in a previous communication (8).

Density gradient centrifugation. Tissue culture supernatant fluids with HCV were adjusted to the desired density by dropwise addition of tris(hydroxymethyl)aminomethane (Tris)-buffered saline, pH 7.2 (2), saturated with cesium chloride (E. Merck AG, Darmstadt, Germany; no. 2041). We pipetted 5 ml of this material into Lusteroid cellulose tubes (Beckman Instruments, Inc., Fullerton, Calif.; no. 3934) and covered it with mineral oil. We centrifuged three tubes in a swinging-bucket titanium rotor (SW 65 K) for 15 to 24 hr at 35,000 to 40,000 rev/min (100,000 to 130,000 $\times g$) at a temperature of 4 C with the use of a Spinco model L ultracentrifuge.

The procedure employed for collecting the fractions has been already described (8). Usually, we collected 7 drops per fraction. The first and the last two fractions of each gradient were discarded.

Density was determined by measuring the refractive indices in an Abbe 3 L refractometer (Carl Zeiss, Oberkochen, Germany) using the formula of Ifft et al. (9). The reliability of this method was controlled by weighing standard samples.

RESULTS

We first studied the influence of CsCl on the infectivity of HCV. Tris-buffered saline (2) saturated with CsCl was added dropwise to tissue culture fluid with HCV (strain München 1) until a density of 1.19 g/ml was obtained for the mixture. A control tube contained saline without CsCl and virus material in equal amounts. Both tubes were stored at 4 C for 24 hr, and then were titrated by the END method. In both samples, $10^{4.2}$ ID₅₀ per 0.2 ml was found. Similar results were obtained with infectious pig serum (strain Behring) titrated by the HEIC procedure after exposure to CsCl at a density of 1.34 g/ml. These results demonstrate that CsCl under stationary conditions does not measurably affect HCV infectivity.

In a preceding study (8), in which piglets were inoculated, we reported the susceptibility of HCV to treatment with chloroform. This property of HCV was also detected with the HEIC method. After treatment of a HCV suspension containing $10^{4.6}$ ID₅₀ per ml with chloroform, less than $10^{1.4}$ ID₅₀ per ml could be found.

Tris-buffered saline saturated with CsCl was added to fluids collected from HCV-infected ST

FIG. 1. Distribution of infectivity of hog cholera virus after equilibrium centrifugation in CsCl density gradients, tested by the END (●) and HEIC (○) method.

cultures (strain München 1) in amounts yielding densities of 1.14, 1.17, and 1.19, respectively. The samples were centrifuged for 20 hr at 35,000 rev/min ($100,000 \times g$). After centrifugation, infectivity titers of the fractions were determined by the END method. The resulting distribution of infectivity is shown in Fig. 1. Similar curves were obtained in seven additional experiments, using either virus strain ALD grown in PK cells or infectious pig serum (strain Behring). The HEIC titration method was used in three of these experiments. The highest infectivity titers were found at a density of 1.16 g/ml; however, a considerable amount of infectious virus was distributed over fractions with densities between 1.17 and 1.20 g/ml.

With densities between 1.17 and 1.18, three fractions were pooled and titrated in the presence of HC-hyperimmune serum (Italy) and normal pig serum, respectively, both diluted to 1:50. The infectivity titer in the presence of hyperimmune serum was $<10^{2.2}$, and that in the presence of normal serum was $10^{4.6}$, thus indicating that the infectious agent, with the above-mentioned buoyant density, is specifically neutralized by a HC-hyperimmune serum.

DISCUSSION

A buoyant density value of 1.24 g/ml has been determined for most of the chloroform-susceptible ribonucleic acid viruses (Table 1). Recently, vesicular stomatitis (17, 28) and rabies (20) viruses have been investigated and found to have densities of 1.19 to 1.22 and 1.20 g/ml, respectively. For the group of murine leukemia viruses

and Rous sarcoma virus, densities of 1.16 and 1.16 to 1.19 g/ml, respectively, have been reported. In connection with our study, the results of Crawford (3), showing density heterogeneity for Rous sarcoma virus, are especially interesting. We obtained similar results with HCV; the highest infectivity titers were registered at a density of 1.16 g/ml; a second peak, more or less pronounced in different experiments, appeared at a density of 1.18 g/ml.

To account for its low density, the HC virus can be assumed to have a high lipid content, since proteins alone have densities of about 1.30 g/ml. An explanation for the observed differences may be found in the structure of the HC virion. Sensitivity to chloroform gives a hint that the HC virion has an envelope containing lipids; variations in the amount or composition of this lipid-containing outer membrane may lead to the observed variations in density.

Preliminary results obtained with bovine viral diarrhea virus show that buoyant density of this virus is lower than 1.18 (Hafez, *personal communication*).

ACKNOWLEDGMENTS

This investigation was supported by the Bundesministerium für Ernährung, Landwirtschaft und Forsten, Bonn, Germany.

We are grateful to K. Dräger, G. Korn, and H. Mahnel for supplying the hog cholera and Newcastle disease virus strains. D. L. Nardelli and H. Plonait kindly provided immune and colostrum-deprived piglet sera, respectively. We are indebted to Hilma Dubenkropp for skillful technical assistance. Sincere appreciation is expressed to M. Mussgay for helpful discussions and valuable criticism.

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TABLE 1. Buoyant densities of ribonucleic acid viruses susceptible to chloroform

Virus	Buoyant density	Reference
	g/ml	
Influenza A.....	1.24	1
Newcastle disease.....	1.28	24
Parainfluenza 2.....	1.24	10
Measles.....	1.24-1.25	22
Arbo A (Sindbis).....	1.24	19
Arbo B (Dengue 2).....	1.24	26
Vesicular stomatitis.....	1.19-1.22	17, 28
Rabies.....	1.20	20
Murine leukemia		
Rauscher.....	1.16	23
Moloney.....	1.16	23
Rous sarcoma.....	1.16	23
	1.18	3
	(1.16-1.19)	
Bovine viral diarrhea...	1.18	(Hafez, <i>personal communication</i>)

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