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INDUCEMENT OF CYTOPATHIC CHANGES AND PLAQUE FORMATION BY PORCINE HAEMAGGLUTINATING ENCEPHALOMYELITIS VIRUS

K. SATO, Y. INABA, Y. MIURA and S. TOKUHISA
National Institute of Animal Health, Tsukuba, Ibaraki 305 (Japan)
M. MATUMOTO
Kitasato Institute, Minato-ku, Tokyo 108 (Japan)

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ABSTRACT

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ESK cells were shown to be a good medium for propagating the 67N strain of porcine haemagglutinating encephalomyelitis virus, although no cytopathic effect was observed. The virus induced a readily recognizable cytopathic effect in ESK cells, when a non-cytotoxic amount of diethylaminoethyl-dextran (DEAE-dextran) was incorporated in the culture medium. Based on this finding, a sensitive, practical assay method for the virus was developed. When DEAE-dextran was incorporated in the agar overlay medium, 67N virus formed plaques in ESK cell monolayers. The cytopathic effect as well as the plaque formation were specifically inhibited by antisera against the virus. Neutralization tests were developed on the basis of these findings. Neutralization and haemagglutination-inhibition tests on swine serum samples indicated a wide dissemination of haemagglutinating encephalomyelitis virus or antigenically-related viruses in Japanese pigs.

INTRODUCTION

Mengeling et al. (1972) isolated a haemagglutinating virus (strain 67N) in primary cultures of embryonic pig kidney cells from the nasal cavity of an apparently healthy feeder pig in the United States. This virus was subsequently shown to be the porcine haemagglutinating encephalomyelitis virus (HEV) which had been isolated previously in Canada (Greig et al., 1962; Greig and Girard, 1963).

The HEV (67N strain) multiplied, but failed to induce readily recognizable cytopathic effect in primary cultures of swine embryonic kidney cells. Therefore, the virus has been assayed in these cultures by a rather cumbersome method in which the presence of virus infection was determined by haemadsorption with chicken erythrocytes or by microscopic examination

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for syncytia of cultures stained with May—Grünwald—Giemsa stain (Mengeling et al., 1972).

Recently we observed that the 67N strain replicated readily, but induced no cytopathic effect in ESK cells, a continuous cell line from swine embryo kidney (Sugimori et al., 1969). However, the strain was shown to induce cytopathic effect in ESK cells, when a non-cytotoxic amount of diethylaminoethyl-dextran (DEAE-dextran) was incorporated in the culture medium, thus providing a sensitive, practical assay method. The method was readily adapted to a neutralization test. Furthermore, the strain formed plaques in ESK cells under an agar overlay medium containing DEAE-dextran. This paper describes these observations.

MATERIALS AND METHODS

Cell cultures

ESK cells, a stable cell line derived from swine embryo kidney (Sugimori et al., 1969), were used. The cell line was kindly supplied by Dr. T. Sugimori, National Institute of Animal Health, Japan. Cells were grown in Eagle's minimum essential medium (MEM) containing 10% inactivated calf serum, 10% tryptose phosphate broth (TPB) (Difco), 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1 μg ml^{-1} fungizone. Monolayer cultures were prepared in 11 × 100 mm tubes by seeding with 1.25×10^5 cells suspended in 0.5 ml growth medium and incubating at 37° C in a stationary rack. After removal of the culture fluid and washing with MEM, cultures were inoculated with 0.1-ml volumes of virus suspension, incubated at 37° C for 60 min for virus adsorption, fed with 0.5 ml of maintenance medium and incubated at 37° C in a roller drum. The maintenance medium was MEM containing 10% TPB, 0.05% yeast extract, 0.5% sodium glutamate, 0.1% glucose and antibiotics.

Virus

Strain 67N of porcine HEV (Mengeling et al., 1972) was kindly supplied by Dr. K. Hirai, Gifu University, Japan, and passaged twice in ESK cells before being used in the present study.

Diethylaminoethyl-dextran (DEAE-dextran)

A stock solution of DEAE-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) containing 1 mg ml^{-1} was made in distilled water, sterilized by autoclaving and stored at 4° C until use.

Infectivity assay

The following method was established on the basis of the results obtained in the present study. Serial decimal dilutions of the test sample were made in maintenance medium and each dilution was inoculated in 0.1 ml volumes into 3 tube cultures of ESK cells. The inoculated cultures were incubated in a roller drum at 37°C for 7 days. The maintenance medium contained 50 $\mu g\ ml^{-1}$ of DEAE-dextran. At the end of incubation, the cultures were examined for cytopathic effect and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated by the method of Kärber.

Plaque formation

Monolayer cultures of ESK cells prepared in petri dishes, 55 mm in diameter, were inoculated with 0.2-ml volumes of virus diluted in maintenance medium containing 50 $\mu g\ ml^{-1}$ DEAE-dextran and antibiotics. After virus adsorption at 37°C for 60 min, the cultures were washed 3 times with MEM and covered with 6 ml of agar overlay medium. The overlay medium was 0.8% LGT agar (Miles, U.S.A.) in MEM containing 50 $\mu g\ ml^{-1}$ DEAE-dextran and antibiotics. The cultures were incubated in an atmosphere of 5% CO_2 in air at 37°C for 4 days and stained by incubating overnight at 37°C under a second overlay medium containing 100 $\mu g\ ml^{-1}$ iodonitrotetrazolium violet (Sigma, U.S.A.). The infectious titre was expressed in plaque-forming units (PFU).

Antisera to HEV

Antisera against the 67N strain were prepared in rabbits. Supernatant culture fluid, from ESK cells infected with the 67N strain, was centrifuged at 100 000 *g* for 2 h and the resulting pellets were dissolved in 0.01 volume of phosphate-buffered saline (PBS) at pH 7.2. The animals were inoculated intravenously with the virus suspension and 3 weeks later intramuscularly and subcutaneously with an equal-volume mixture of the virus suspension and Freund's complete adjuvant. Serum was obtained 4 weeks later. The sera were inactivated at 56°C for 30 min and stored at −20°C until use.

Neutralization (NT) test

Serial twofold dilutions of the serum inactivated at 56°C for 30 min were made with maintenance medium containing 50$\mu g\ ml^{-1}$ DEAE-dextran. In some experiments serial fourfold dilutions were used. Each serum dilution was mixed with an equal volume of maintenance medium containing 200 $TCID_{50}$/0.1 ml of virus and incubated at 37°C for 60 min. The virus—serum mixtures were then inoculated in 0.1-ml volumes into tube cultures of ESK cells, using 2 tubes for each dilution. After incubation at 37°C for 60 min,

the inoculated cultures were fed with 0.5 ml of maintenance medium containing 50 μg ml^{-1} of DEAE-dextran, incubated in a roller drum at 37° C for 7 days, and examined for any cytopathic effect. The antibody titre was expressed as the reciprocal of the highest serum dilution which showed complete inhibition of cytopathic effect in at least one of the two tubes.

Haemagglutination (HA) test

Haemagglutinin titres were determined by adding 0.025 ml of a 0.5% suspension of washed chicken erythrocytes in PBS to 0.05 ml of each of twofold dilutions of virus. The mixtures were examined after 60 min of incubation at 22° C. The titre was expressed as the maximum virus dilution that caused HA.

HA-inhibition (HI) test

This was carried out by the microtitre method (Sato et al., 1977). The serum was inactivated at 56° C for 30 min and treated with kaolin to remove non-specific inhibitors and with packed chicken erythrocytes to remove antibodies to the cells. The HI antibody titre was expressed as the reciprocal of the highest serum dilution which showed complete inhibition of HA with 4 units of haemagglutinin.

RESULTS

Cytotoxic effect of DEAE-dextran on ESK cells

Experiments were done to determine the toxic concentrations of DEAE-dextran for ESK cells. Tube cultures of ESK cells were fed with 0.5 ml of maintenance medium containing various amounts of DEAE-dextran, and incubated in a roller drum at 37° C for 7 days. The presence of DEAE-dextran in concentrations up to 50 μg ml^{-1} was not injurious to ESK cells, whereas 60 μg ml^{-1} or higher concentrations induced cytotoxic effects consisting of rounding, granulation and eventual disintegration of the cells. These changes began to appear within 24 h and almost all the cells were damaged at the end of incubation in the presence of 100 μg ml^{-1} of DEAE-dextran, while the changes appeared after 2 and 3 days of incubation and 70 and 40% of the cells were destroyed in 7 days of incubation in cultures containing DEAE-dextran at 80 and 60 μg ml^{-1}, respectively.

Development of cytopathic effect of HEV with DEAE-dextran

The 67N strain multiplied, but induced no cytopathic effect in ESK cells. However, the strain replicated with readily recognizable cytopathic effect in these cells in the presence of nontoxic amounts of DEAE-dextran. The cytopathic effect began to appear 3 days after infection and consisted of

cell rounding, fusion and eventual disintegration of the cell sheets (Fig. 1 a,b). Thus, the infectivity of the virus could readily be assayed by using the cytopathic effect as the criterion for virus infection.

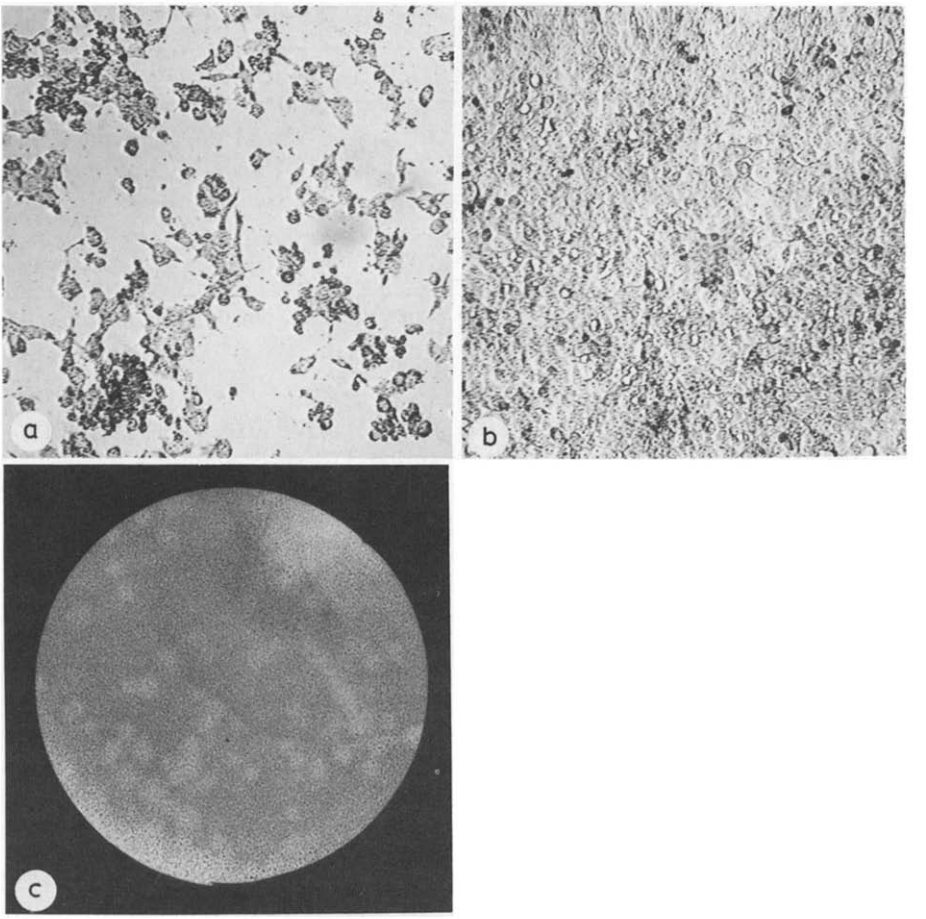

Fig. 1. Cytopathic effect; 5 days after virus infection (a), uninfected control (b). Plaques on ESK cell monolayer (c).

Table I shows the results of such infectivity assays in the presence of various concentrations of DEAE-dextran. In these experiments serial decimal dilutions of a virus suspension were made in maintenance medium containing 10—50 μg ml^{-1} of DEAE-dextran and each dilution was inoculated in 0.1-ml volumes into 3 tube cultures of ESK cells. After virus adsorption at 37° C for 60 min, the inoculated cultures were fed with 0.5 ml of maintenance medium containing the same amount of DEAE-dextran as used for virus dilution, and incubated in a roller drum at 37° C for 7 days. At the end of

TABLE I

Titration of strain 67N of HEV in ESK cell cultures incubated with various concentrations of DEAE-dextran

DEAE-dextran in the medium (μg)	Infectivity[a] determined by	
	CPE	HA
50	5.5	5.5
40	4.8	5.5
30	4.2	5.5
20	4.5	5.5
10	4.2	5.5
0	<0.5	5.5

[a]Infectivity is shown as log ($TCID_{50}$ 0.1 ml^{-1}) which was determined by cytopathic effect (CPE) or by haemagglutinating activity (HA).

incubation, the cultures were examined for cytopathic effect and the $TCID_{50}$ titre was calculated. The cultures containing DEAE-dextran at 10—50 μg ml^{-1} developed cytopathic effect and gave $TCID_{50}$ titres of $10^{4.2}$—$10^{5.5}$ 0.1 ml^{-1}. The titre at 50 μg ml^{-1} DEAE-dextran was the highest. At the end of incubation each tube received 0.2 ml of 0.5% chicken erythrocytes in PBS and was examined for haemagglutination. Haemagglutination was observed in all tubes inoculated with virus up to 10^{-5} dilution irrespective of the presence of DEAE-dextran, but not in those inoculated with 10^{-6} and 10^{-7} dilutions (Table I). On the basis of these results, the method of infectivity assay was determined as described in the materials and methods section.

Replication of HEV in ESK cells

Two sets of tube cultures of ESK cells were inoculated with the virus at an input multiplicity of infection (m.o.i.) of 10 $TCID_{50}$. The cultures were fed with 0.5 ml of maintenance medium and incubated at 37°C in a roller drum. The maintenance medium contained 50 μg ml^{-1} of DEAE-dextran in one set of cultures, but no DEAE-dextran in the other set. At intervals, the culture fluid was harvested from 2 cultures of each group, pooled and assayed for infectivity after centrifugation at 1500 *g* for 10 min. Another pair of cell culture sets were infected at an input m.o.i. of 0.001 $TCID_{50}$ and processed in the same manner as in the above experiment.

The results are illustrated in Fig. 2. In the cultures infected with the heavy virus dose, the infectivity began to rise 6 h post-infection, reached a plateau of about $10^{5.5}$ $TCID_{50}$ 0.1 ml^{-1} at 18 h and remained almost constant until 84 h with subsequent gradual decline. No cytopathic effect was observed. In the presence of DEAE-dextran, however, cytopathic

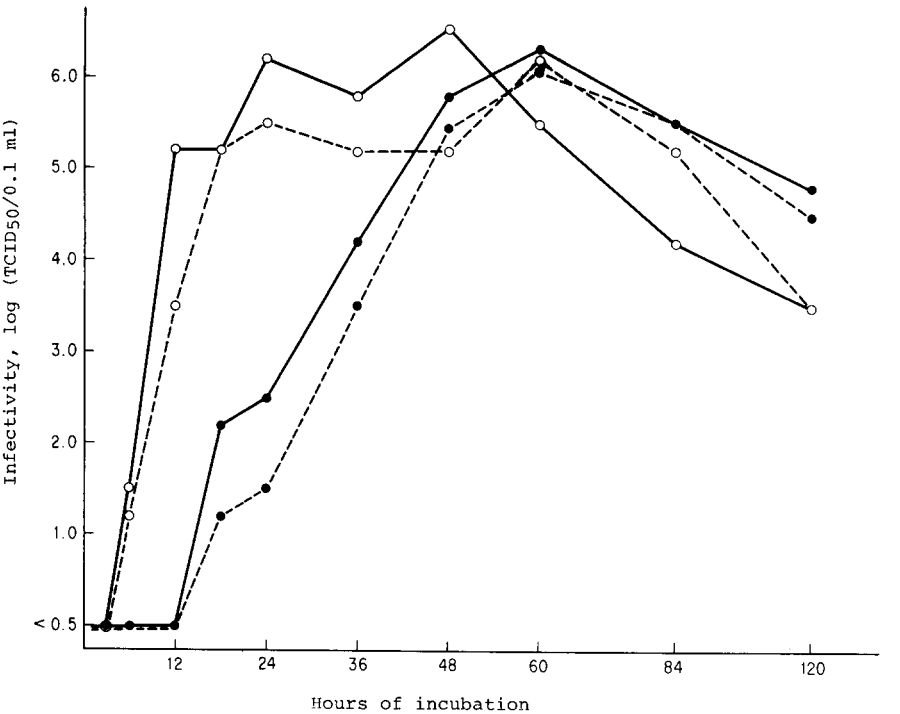

Fig. 2. Replication of strain 67N of HEV in ESK cells in the presence and absence of 50 μg ml^{-1} DEAE-dextran. o——o m.o.i.: 10 $TCID_{50}$/cell, DEAE-dextran (+); o- - -o m.o.i.: 10 $TCID_{50}$/cell, DEAE-dextran (–); •——• m.o.i.: 0.001 $TCID_{50}$/cell, DEAE-dextran (+); • - - -• m.o.i.: 0.001 $TCID_{50}$/cell, DEAE-dextran (–).

effect began to appear 3 days post-infection and the cell sheet was completely destroyed after 7 days of incubation. The virus titre rose about one log-unit higher and began to decline earlier than in the cultures with no DEAE-dextran. In the cultures infected with the small virus dose, the virus titre began to rise later, and rose more slowly than in the cultures with the heavy inoculum, although there was little difference in the level of plateau between these culture groups. In the cultures infected with the small inoculum and incubated with DEAE-dextran, cytopathic effect began to appear 5 days post-infection and the cell sheet was completely destroyed within 9 days. DEAE-dextran somewhat enhanced the virus growth.

In another set of ESK cell cultures infected at an input m.o.i. of 0.001 $TCID_{50}$ and incubated with 50 μg ml^{-1} DEAE-dextran, the HA titre of the culture fluid was 8, two days post-infection, reached 32 at 5 days and remained constant until 7 days, while the virus growth was similar to that shown in Fig. 2.

Specificity of cytopathic effect

The cytopathic effect observed in infected ESK cultures incubated with DEAE-dextran was specifically inhibited by rabbit antisera against the 67N virus (Table II). These antisera were also positive for HI antibody to the virus. Pre-immunization sera were invariably negative for both NT and HI antibodies. These results confirm the specificity of the cytopathic effect.

TABLE II

Neutralization (NT) and haemagglutination-inhibition (HI) tests of strain 67N of HEV with rabbit antisera against the virus

Antiserum	NT titre	HI titre
Rabbit no. 1 pre-	<2	<10
post-	65 500	320
Rabbit no. 2 pre-	<2	<10
post-	131 000	1280

Plaque formation

The 67N strain formed plaques in ESK cell monolayers under an agar overlay medium containing 50 μg ml^{-1} of DEAE-dextran. The plaques were clearly defined, circular and 1.5 to 2 mm in diameter (Fig. 1c). The infectivity titre in PFU was comparable to the $TCID_{50}$ titre obtained in tube cultures. The plaque formation was specifically inhibited by rabbit antisera against the virus.

NT and HI antibodies to HEV in Japanese pigs

Serum samples collected from 60 adult pigs at an abattoir in Ibaraki Prefecture over the period from November 1979 to January 1980 were tested for NT and HI antibodies against the 67N strain. The results are shown in Fig. 3. Both antibodies were detected in 44 (73.3%) of the 60 specimens and the remaining 16 specimens were negative in both tests. NT titres of individual pigs were closely correlated with their HI titres; the correlation coefficient was 0.59 ($P < 0.01$).

DISCUSSION

In the present study, ESK cells were shown to be a good medium for the propagation of the 67N strain of HEV, although no cytopathic effect was

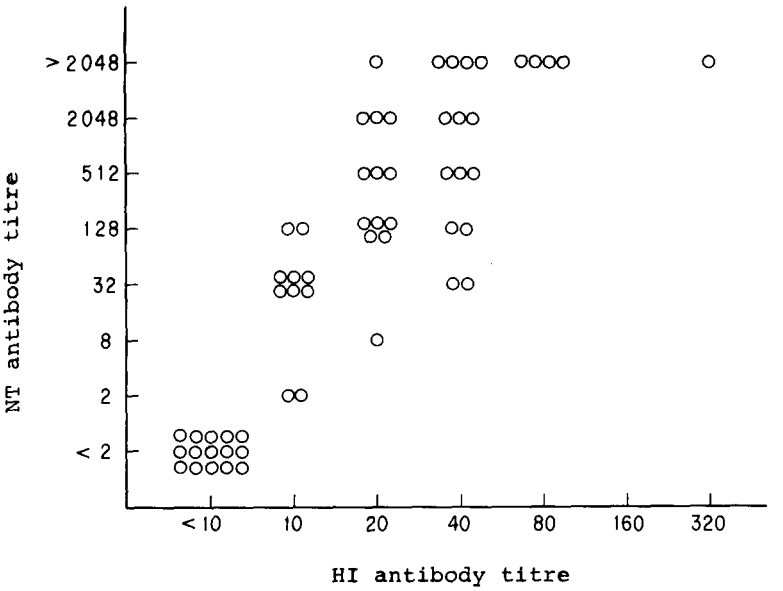

Fig. 3 Neutralizing (NT) and haemagglutination-inhibiting (HI) antibody titres to strain 67N of HEV in normal adult swine sera.

observed. The virus induced a readily recognizable cytopathic effect in these cells, when a non-cytopathic amount of DEAE-dextran was incorporated in the culture medium. Based on this finding, a sensitive, practical assay method for the virus was developed. This is particularly important since the virus has been assayed in tissue cultures by rather cumbersome methods (Mengeling et al., 1972).

The infectivity of polio (Pagano and Vaheri, 1965), rubella (Vaheri et al., 1967), rabies (Kaplan et al., 1967) and varicella—zoster viruses (Sasaki et al., 1981) has been reported to be enhanced by DEAE-dextran. The enhancing effect of DEAE-dextran has also been reported with human coronavirus (Bradburne and Tyrrell, 1969) and mouse hepatitis virus (Takayama and Kirn, 1976; Hirano et al., 1978).

Although the replication of the 67N virus in ESK cells was only slightly enhanced by DEAE-dextran, the development of cytopathic effect by the virus in the presence of DEAE-dextran was impressive. The mechanism of the induction of cytopathic effect by DEAE-dextran is not known, but obviously it is not caused by enhanced viral growth.

When DEAE-dextran was incorporated in the agar overlay, HEV formed plaques in ESK cell monolayers. This finding provides a sensitive assay method and the technique lends itself to the isolation of viral clones.

The cytopathic effect as well as the plaque formation induced in ESK cells by the 67N virus in the presence of DEAE-dextran was specifically inhibited by antisera against the virus. Based on these findings neutralization tests were developed.

NT and HI tests on swine serum samples indicated a wide dissemination of HEV or antigenically-related viruses in Japanese pigs, confirming the previous report (Sato et al., 1980).

The assay methods developed in this study for infectivity of the 67N virus and for neutralizing antibody to the virus could find a wide application in studies of the virus and its infections.

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