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IN VITRO TRANSFORMATION OF HAMSTER CELLS BY INFECTIOUS CANINE HEPATITIS VIRUS

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INTRODUCTION

A number of adenoviruses have been proven oncogenic for newborn hamsters. Based upon their oncogenic properties, the human adenoviruses have been divided roughly into 3 subgroups: the highly oncogenic adenoviruses, the weakly oncogenic and the non-oncogenic.

But recently, FREEMAN et al. (1967)², NOORDAA (1968), and McALLISTER et al. (1969) demonstrated the capacity of human adenovirus types 1, 2 and 5, hitherto not known to be oncogenic types, to cause transformation in tissue cultures.

In a previous report⁵, the authors demonstrated the oncogenic potential for newborn hamsters of infectious canine hepatitis virus (ICHV) (canine adenovirus). But the frequency of tumor production was low.

To make more clear the oncogenic properties of ICHV, an attempt was made by the authors to investigate in vitro transformation of hamster cells by ICHV. This paper describes the results.

MATERIALS AND METHODS

Virus strains Eleven strains of ICHV were used. Details of the sources and serological properties of the strains were described previously⁴.

These viruses were grown in dog kidney cell (DKC) cultures. A monolayer of DKC showing complete cytopathic effect (CPE) was harvested by scraping the cells into medium. The cell suspension was frozen and thawed 3 times and clarified by centrifugation at 3,000 rpm for 30 min. The supernatant fluid was used as stock virus. Titration of the virus was carried out using DKC.

Hamster cells Hamster embryo cells were prepared by trypsinization of near-term embryos and were suspended in growth medium with a concentration of about 2×10^5 cells per ml. Petri dishes (5 cm in diameter) and rubber-stoppered small bottles (3×5×3 cm) containing cover slips were seeded with 5 ml of the cell suspension, incubated at 37°C in air, and air containing 5% CO₂, respectively.

Usually primary or secondary cultures were used. In one experiment, cultures of the 4th passage of baby hamster kidney cells were used.

Media Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate, 0.3% tryptose phosphate broth (Difco) and antibiotics was used as the basal medium. The medium supplemented with 10% calf serum was used as the growth medium. For transformation experiments, the basal medium with a reduced calcium chloride content, 0.1 mM, (referred to as basal medium (-)) was used.

Transformation assays Confluent monolayers of hamster cells (approximately $10^6 \sim 2 \times 10^6$ cells/culture) in petri-dishes and in small bottles were rinsed twice with phosphate buffered saline and were inoculated with 0.5 ml of the virus suspension for 2 hr at 37°C. After the adsorption period, the cultures were fed with 5 ml of the basal medium (-) containing 10% calf serum. The cultures were maintained for the first month by feeding the cells every other day with the same medium, and thereafter were fed twice a week with the medium containing 7% calf serum. The cultures were observed for at least 2 months.

Tumorigenicity To determine the tumor-producing potential of the transformed cells, the cultures were trypsinized and were inoculated subcutaneously into baby hamsters. The animals were observed for at least 2 months.

Fluorescent antibody (FA) study For detection of T antigen of ICHV in the transformed cells, an indirect FA test was done. For this purpose serum from the hamsters bearing a transplanted tumor induced by ICHV and fluorescein-conjugated goat anti-hamster serum were used.

RESULTS

1 Transformation of hamster cells by ICHV

A preliminary experiment showed that typical CPE occurred in hamster cells inoculated with a relatively large dose of the virus (input multiplicity of 2 TCID₅₀ per cell or more). In such cases, it was difficult to maintain these cultures for a month even if the change of medium was made daily. Therefore we used the input multiplicity of about 1 TCID₅₀ per cell or less.

Using various kinds of hamster cells, 6 experiments were performed. Four of them succeeded in maintaining the cultures for a month, which are described below.

Table 1 shows the results of transformation of primary hamster embryo cells by the virus. Transformed focus was observed in cultures of bottles, inoculated with strains NIII-E and Otaru (fig. 1). The foci revealed 37 days after inoculation, against a fibroblastic background, morphologically altered areas consisting of small epitheloid cells forming distinct domed foci (figs. 2 & 3). These foci were similar to those previously described in hamster embryo cells transformed by oncogenic human adenoviruses.

No transformed focus other than CPE was observed in the cultures inoculated with strains FD and Woc-4 which have been proven oncogenic for newborn hamsters⁵⁾. The focus did not develop in uninoculated control cultures.

Table 2 shows the data of another transformation experiment using primary hamster embryo cells, in which the cultures in bottles were subcultured 24 hr after inoculation into new bottles and dishes.

All the cultures inoculated with strain FD at an approximate input multiplicity of

TABLE 1 *Transformation of primary hamster embryo cells by ICHV (Exp. 1)*

STRAINS	APPROX. VIRUS INPUT *1	SMALL BOTTLE			PETRI DISH		
		No. used	No. of foci per bottle		No. used	No. of foci per dish	
FD	1	3 (12) *2	0 *3, 0, 0		3 (20)	0, 0, 0	
Woc-4	1	1 (2)	0		2 (5)	0, 0	
NIII-E	1	2	1, 0		4 (5)	0, 0, 0, 0	
Otaru	1	2	1, 0		4 (5)	0, 0, 0, 0	
Control	—	3	0, 0, 0		4	0, 0, 0, 0	

*1 TCID₅₀/cell

*2 3 (12) means that of 12 bottles, 3 were actually used for the experiments, and others in which the cells were detached from glass due to CPE caused by ICHV multiplication were omitted.

*3 Foci were counted 60 days after virus inoculation.

These footnotes are also used in the following tables.

TABLE 2 *Transformation of primary hamster embryo cells by ICHV (Exp. 2)*

STRAINS	APPROX. VIRUS INPUT	SMALL BOTTLE			PETRI DISH		
		No. used	No. of foci per bottle		No. used	No. of foci per dish	
FD	0.5	0 (4)	—		0 (4)	—	
Woc-4	0.5	3 (4)	1, 0, 0		4	3, 1, 0, 0	
FS	1.0	4	0, 0, 0, 0		4	2, 0, 0, 0	
P-2	1.0	4	1, 0, 0, 0		4	1, 0, 0, 0	
Control	—	4	0, 0, 0, 0		4	0, 0, 0, 0	

Primary hamster embryo cells cultivated in bottles were inoculated with ICHV and, after 24 hr, were subcultured into small bottles and petri dishes.

0.5 TCID₅₀ per cell showed complete CPE. In contrast, transformed foci appeared in 1 of 3 cultures in bottles and 2 of 4 cultures in dishes inoculated with strain Woc-4. Strains FS and P-2 also transformed the hamster cells in this system.

In the experiment shown in table 3, secondary cultures of hamster embryo cells were used. Transformation occurred in many dishes. Six of 7 strains used showed transforming ability.

Cultures of the 4th passage of baby hamster kidney cells were used in the experiment shown in table 4. Of the 3 virus strains only P-2 formed a focus in 2 of 4 cultures.

In table 5, the results of 4 experiments described above were summarized.

TABLE 3 *Transformation of secondary hamster embryo cells by ICHV (Exp. 5)*

STRAINS	APPROX.	NO. OF PETRI DISHES USED	NO. OF FOCI		
	VIRUS INPUT		PER DISH		
N-II	1.0	4	2,	1,	1, 0
N-III	0.1	3	1,	1,	0
Nakano	1.0	3	2,	1,	1
Otaru	0.1	3	2,	1,	0
P-2	1.0	3	3,	1,	0
C-I	1.0	3	0,	0,	0
N-IV	1.0	3	2,	1,	0
Control	—	3	0,	0,	0

TABLE 4 *Transformation of baby hamster kidney cells in the 4th passage by ICHV (Exp. 6)*

STRAINS	APPROX.	NO. OF PETRI DISHES USED	NO. OF FOCI		
	VIRUS INPUT		PER DISH		
FS	1	4	0,	0,	0, 0
P-2	1	4	1,	1,	0, 0
C-I	1	3	0,	0,	0
Control	—	3	0,	0,	0

Four transformed foci were observed in 4 of 19 inoculated cultures in bottles which could be maintained for more than a month; while 29 foci developed in 20 of 59 dishes cultivated in a 5% CO₂ incubator.

Nine of 11 ICHV strains transformed the hamster cells.

2 Fluorescent antibody study

Two transformed foci, caused by strains NIII-E and Otaru, on coverslips in small bottles were stained with tumor bearing hamster serum by the indirect FA test.

Almost all cells in the foci showed specific nuclear fluorescence with ICHV tumor antiserum. Morphologically, the nuclear staining patterns were similar to those observed in the cultured cells of the ICHV induced hamster tumor⁶).

3 Tumorigenicity of the ICHV transformed hamster cells

In each experiment, the cell cultures inoculated with the same strain were pooled and

TABLE 5 *Transformation of hamster cells by ICHV:
Summary of the experiments 1, 2, 5 and 6*

STRAINS	SMALL BOTTLE			PETRI DISH		
	No. used	No. of positive	Total No. of foci	No. used	No. of positive	Total No. of foci
FD	3 (16)	0	0	3 (24)	0	0
Woc-4	4 (6)	1	1	6 (9)	2	4
N-II	—			4	3	4
N-III	—			3	2	2
NIII-E	2	1	1	4 (5)	0	0
Nakano	—			3	3	4
Otaru	2	1	1	7 (8)	2	3
FS	4	0	0	8	1	2
P-2	4	1	1	12	5	7
C-I	—			6	0	0
N-IV	—			3	2	3
Total	19 (34)	4	4	59 (85)	20	29
Control	7	0	0	14	0	0

were injected subcutaneously into hamsters with approximately 10^6 cells per animal. In the experiments 2 and 5, the cells were also used for preparation of secondary cultures.

The cell cultures used in experiment 1 were inoculated into 20 13-day-old hamsters. Four hamsters were used for each strain and control. Of the 4, 2 each were inoculated with the cell cultures from small bottles and petri dishes. A tumor developed in only one hamster 98 days after inoculation with the cells in bottles transformed by strain NIII-E.

TABLE 6 *Tumorigenicity of the ICHV transformed hamster cells shown in experiment 2*

STRAINS	CELL CULTURES IN BOTTLE		CELL CULTURES IN DISH	
	Original *1	Subcultured *2	Original	Subcultured
Woc-4	0/2 *3	3/3	1/2	3/3
FS	0/1	0/2	0/2	0/2
P-2	0/2	0/2	0/2	0/2
Control	0/1	0/2	0/2	0/2

*1 Cell cultures inoculated with either ICHV or medium as control

*2 Original cell cultures were subcultured.

*3 No. of tumored hamster/No. of inoculated

*4 These inoculated hamsters died accidentally before weaning.

The cell cultures used in experiment 2 were inoculated into 14 27-day-old hamsters. The results are shown in table 6. Only one hamster developed a tumor 37 days after inoculation with the cells in dishes transformed by strain Woc-4. This tumor was confirmed to contain the ICHV T antigen by the CF test. In addition, the serum from this animal had a CF titer of 1:8 when tested against ICHV induced tumor antigen. The other 13 animals, including 3 control, did not develop a tumor within the 90 day observation period.

Contrary to the original cultures described above, subcultured cells transformed by strain Woc-4 induced tumors in all 6 hamsters (tab. 6). The hamsters used were 7 days old, and the tumors developed within 3 weeks with the subcultured cells, either in bottles or dishes. All the sera from tumored hamsters contained a CF antibody reactive against the ICHV T antigen. The other 12 baby hamsters inoculated with subcultured cells transformed by the other strains and those of control cells died accidentally before weaning.

The cell cultures used in experiment 5 were also inoculated into 30-day-old hamsters. One hamster was used for each strain. A tumor developed in 2 animals, on the 41st and 45th days, inoculated with the cells transformed by strains N-IV and Otaru respectively. The animals inoculated with the subcultured cells did not develop a tumor within 40 days but are now under observation.

Eight baby hamsters inoculated with the cell cultures used in experiment 6 did not develop a tumor within 2 months and are also under observation.

DISCUSSION

The present study was undertaken to investigate whether ICHV transforms hamster cells *in vitro*.

In the ICHV-hamster cell system, the cells inoculated with ICHV at an input multiplicity of 1 TCID₅₀ or more, showed cytolytic ICHV CPE and were difficult to maintain for a long period. The fact is somewhat different from that of the oncogenic human adenovirus-hamster cell system. Therefore, we used a relatively small dose of inoculum such as 1, 0.5 and 0.1 TCID₅₀ per cell. The hamster cells we used were various as described in RESULTS.

Strains FD and Woc-4, proven oncogenic for newborn hamsters previously⁵⁾, were chiefly used in experiments 1 and 2. Strain FD caused cytolytic CPE even at an input multiplicity of 0.5 TCID₅₀. In this case, only 3 of 16 cultures in bottles and 3 of 24 cultures in dishes could partially be maintained for more than a month. However no transformed focus was developed in these cultures within 3 months. It may be due to scarcity of susceptible cells by CPE.

Strain Woc-4 on the other hand, produced only partial CPE at an input multiplicity of 0.5 TCID₅₀. The cultures could be maintained for more than a month and in some of them transformed foci were developed.

CASTO reported that the transfer of inoculated cells into new plates appears to be necessary for the rapid demonstration of adenovirus-transformed cell foci. We applied this method in experiment 2 with somewhat better results than those

of experiment 1, where no transfer was applied. But as shown in experiment 5 (tab. 3), a relatively high transformation rate was obtained in the secondary cultures of hamster embryos without transfer. Therefore, transformation efficiency seems to be dependent on many other factors as reported by SCHELL et al.

In experiment 6 (tab. 4), we used the 4th tissue culture passage which consisted mainly of fibroblastic cells as described by VONKA et al., but particularly good results were not obtained.

With regard to the atmosphere in which the culture was prepared, transformation efficiency was compared in the experiments 1 and 2 between cultures in rubber-stoppered bottles and in dishes in a 5% CO₂ incubator. But no particular difference in frequency of transformation was observed.

From the results of 4 experiments presented here, it was demonstrated that 9 of 11 strains of ICHV had transforming potency for hamster cells. Of the remaining 2 showing no transforming potency, strain FD has been proven oncogenic for newborn hamsters⁵⁾. Therefore, it could be said that, under proper conditions, all strains of ICHV have oncogenic potency.

ICHV T antigen was detected by FA test in 2 foci tested. Development of a tumor was found in a total of 4 out of 40 hamsters inoculated with the transformed cell cultures. One of the tumors was tested and confirmed to contain the ICHV T antigen by a CF test, and the serum from the tumored hamster had a CF antibody reactive against ICHV T antigen. These facts indicate that the transformed foci were caused by ICHV.

An unexpected result of the present study was the low rate of tumor induction by the transformed cells. Recently, McALLISTER et al. (1969) described a similar phenomenon. They failed to develop a tumor in rats with human adenovirus types 1 and 2 which transformed rat embryo cells. They speculated that adenovirus types 1 and 2 may produce strong transplantation antigens in the cells they transformed, thus minimizing their chances of initiating a focus of tumor cells.

In our case, however, the tumor was developed when strain Woc-4 transformed cells was inoculated after subcultivation.

We speculate from this finding that in the case of primary transformed cells, the actual number of transformed cells inoculated per hamster might be too small to produce a tumor; while by transferring these cells into secondary cultures, transformed cells might be selectively increased in their numbers, and consequently tumorigenicity of the secondary cultures was enhanced. With regard to this point, further study will be necessary.

The increase of the multiplicity of infection generally enhanced the rate of transformation in human adenoviruses^{1,3)}. In our case of ICHV it led to cellular

death. To analyse the oncogenic property of ICHV in more detail, we are now trying to transform rat embryo cells, in which no CPE was found even when inoculated with large dose of ICHV.

With regard to rat embryo cells, several workers^{2,7,8)} succeeded in transforming these cells by human adenovirus types 1, 2 and 5, hitherto known as non-oncogenic types. Rat cells may be more sensitive than hamster cells in being transformed by weakly oncogenic viruses.

This is the first report of successful transformation by ICHV.

SUMMARY

Hamster cells were transformed by 9 of 11 strains of ICHV. Transformed cells formed foci of multilayered growth in the monolayer cultures. The foci appeared from 37 to 60 days after inoculation. The transformed cells were found to contain ICHV T antigen by indirect FA test. The rate of transformation was low.

Tumorigenicity of the transformed cells for hamsters was low, confirmed only in 4 out of 40 animals inoculated. However, when the transformed cells were inoculated into hamsters after subcultivation, the development of the tumor was greatly increased. The tumors were confirmed specific for ICHV.

From the data presented, it is shown that ICHV is capable of inducing in vitro transformation of hamster cells.

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EXPLANATION OF PLATE

Fig. 1 Foci (arrow) of transformed hamster cells 60 days after inoculation with ICHV

Giensa staining $\times 1.3$

Fig. 2 A part of the transformed focus

Transformed focus (left half) consisting of small epitheloid cells was easily distinguishable from the normal fibroblastic background (right half).

Photograph was taken 50 days after infection.

Unstained preparation $\times 180$

Fig. 3 The same focus showing figure 2, 60 days after infection

Giensa staining $\times 180$

