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Citation	Japanese Journal of Veterinary Research, 17(1-2), 43-53
Issue Date	1969-06
DOI	https://doi.org/10.14943/jjvr.17.1-2.43
Doc URL	https://hdl.handle.net/2115/1922
Type	departmental bulletin paper
File Information	KJ00002369754.pdf

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INTERFERON-LIKE INHIBITOR DETECTED IN CULTURE FLUIDS OF NORMAL HELA CELLS

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(Received for publication, March 18, 1969)

INTRODUCTION

HeLa cells supported an incomplete growth of infectious canine hepatitis (ICH) virus (KINJO & YANAGAWA, 1967[9]), and such infected HeLa cells showed a persistent low grade infection (KINJO & YANAGAWA, 1967[10]). We assumed an interferon participate in this virus-cell system. Therefore we studied interferon production by ICH virus in HeLa cells.

Contrary to our expectation, we found that culture fluids from normal HeLa cells contained an interferon-like inhibitor against ICH virus when tested in dog kidney cell cultures.

Some properties of this interferon-like inhibitor have been studied and are described in this paper.

MATERIALS AND METHODS

Virus strain The strain Woc-4 of ICH virus was used.

Cell cultures The line of HeLa cells designated as A-HeLa cells was obtained from the Department of Bacteriology, Faculty of Medicine, Hokkaido University and has been maintained more than 10 years in this laboratory. Another line of HeLa cells, designated as B-HeLa cells for convenience, and HeLa-S3, a clonal subline of HeLa cells were obtained from the Hokkaido Institute of Public Health, just prior to use.

The line of A-HeLa cells was used in most of the experiments.

The secondary cultures of dog kidney cells (DKC) were prepared as described previously[9] and were used.

These HeLa cells and DKC were grown in Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate and 10% calf serum, and were maintained in the same medium containing 1～2% calf serum.

Preparation of interferon HeLa cells in the bottle were inoculated with 1 ml of virus material containing about 10^6 $TCID_{50}$ per ml. After 2 hr adsorption at 37°C, the maintenance medium (10 ml) was added.

After 4 days incubation at 37°C, culture fluids were collected and were centrifuged at 3,000 rpm for 30 min. The supernatant fluids were recentrifuged at 36,000 rpm for 90 min.

The final supernatant fluids thus obtained were served as interferon preparations. Control preparations were prepared in the same manner, but normal DKC fluid was inoculated instead of the virus.

Interference assays The cell monolayer of DKC in the small bottle, after the removal of the growth medium, was washed twice with phosphate buffered solution (PBS) (pH 7.4), and was added to 2 ml of the maintenance medium containing 10% of interferon preparation to be tested. After 16~18 hr incubation at 37°C, culture fluids were removed. The DKC were washed once with PBS and were challenged with 0.5 ml of ICH virus materials containing 2×10^3 $TCID_{50}$ per ml. After 2 hr adsorption at 37°C, non-adsorbed virus was discarded, the cells were washed twice with PBS and were added to 5 ml of the maintenance medium. The cultures were then incubated at 37°C for 72 hr. The appearance of the cytopathogenic effects (CPE) in the cultures were scored and virus titers of the fluid were obtained by the same methods described previously[9].

The degree of interference was obtained from the difference between the titers of the challenge virus in the test and control cultures.

Chemical treatments of interferon The methods described by BADER (1962) were used. Solutions at pH 2 were made with 0.05 M KCl and HCl. Interferon preparations were dialyzed against 3 changes of this solution during 20 hr at room temperature. Physiological pH was restored by dialysis against Hanks balanced salt solution for 6 hr.

Trypsion (Difco, 1 : 250) was added to the interferon preparations to give a final concentration of 0.01% and the mixtures were incubated for 4 hr at 37°C.

DNase (Sigma) (final concentration 20 μg/ml) and RNase (Sigma) (final concentration 10 μg/ml) were added to the interferon preparation for 1 hr at 37°C.

RESULTS

1 Demonstration of the interferon-like inhibitor in the culture fluids of normal HeLa cells

Table 1 showed 4 experimental data. In DKC pretreated with the fluids of ICH virus infected HeLa cells, almost the same titer of ICH virus was obtained at 72 hr as in DKC pretreated with fresh maintenance medium alone which had not been exposed to HeLa cells.

However, in DKC pretreated with the fluids of normal HeLa cells almost 2 logs low titer was obtained. The results suggest that the interferon-like inhibitor which could inhibit the ICH virus multiplication in DKC was produced by uninfected, normal HeLa cells.

Hereafter we use the term "interferon-like inhibitor" for the above ICH viral inhibitor present in fluids of normal HeLa cells.

2 Presence of the interferon-like inhibitor in fluid and cellular fractions of normal HeLa cells

The amount of the interferon-like inhibitor in the fluid and cellular fractions of normal HeLa cells was compared. The cellular fraction tested was obtained as follows: After removal of fluids, the cells were washed twice with PBS, scraped from glass with a rubber policeman, and resuspended in original volume of maintenance medium. The cell suspensions were then sonicated in a Kubota's oscilator (KMS-100) at 10 Kc for 5 min and centrifuged

TABLE 1 *Interferon-like inhibitor in culture fluids of normal HeLa cells*

TEST MATERIAL*1	EXP. NO.				MEAN
	1	2	3	4	
Fluids of normal HeLa cells	4.2*3	3.2	3.8	4.2	3.85
Fluids of ICH virus infected HeLa cells	4.8	5.5	6.5	5.8	5.65
Medium control*2	5.2	5.8	6.2	5.8	5.75

*1 Each of 10% test material in the maintenance medium was added to DKC. After 16~18 hr pretreatment at 37°C, the DKC was challenged with ICH virus.
*2 Maintenance medium non-exposed to HeLa cells was used.
*3 Virus infectivity titer expressed as log $TCID_{50}$ per ml at 72 hr after virus challenge
These foot-notes are similarly used in the following tables unless otherwise described.

at 36,000 rpm for 90 min. The supernatant fluids were served as the cellular fraction.

The results, shown in table 2, indicated that an interferon-like inhibitor was present in both fluid and cellular fractions but the amount was larger in the fluid fraction.

TABLE 2 *Presence of interferon-like inhibitor in fluid and cellular fractions of normal HeLa cells*

TEST MATERIAL*	EXP. NO.				MEAN
	1	2	3	4	
Fluid fraction	3.5	3.8	3.2	4.8	3.83
Cellular fraction	4.5	3.8	3.5	4.8	4.15
Medium control	5.2	5.8	6.2	5.8	5.75

* Fluid and cellular fractions were obtained from the cultures of normal HeLa cells. Method for preparation of cellular fraction is described in the text.

3 Effect of cultivation period of HeLa cells on production of the interferon-like inhibitor

To know whether the production of interferon-like inhibitor was influenced by the cultivation period of HeLa cells, the culture fluids were taken at the various days as indicated in table 3 and were tested for the interfering activity to the ICH virus multiplication in DKC.

As shown in table 3, the culture fluids obtained from infected HeLa cells showed almost no interfering activity at any time tested; in contrast, the normal culture fluids showed interfering activity.

TABLE 3 *Effect of cultivation periods of HeLa cells on production of interferon-like inhibitor*

TEST MATERIAL	CULTIVATION PERIODS IN DAYS*					
	1	2	4	7	10	15
Fluids of normal HeLa cells	4.5	3.2	3.5	3.5	4.5	5.5
Fluids of ICH virus infected HeLa cells	5.2	5.5	6.2	5.8	5.5	5.2
Medium control	5.8	5.8	5.5	6.2	5.5	5.8

* At cultivation period 0, the confluent monolayer of HeLa cells was inoculated with ICH virus or with normal DKC fluid instead of the virus, and following adsorption added with fresh maintenance medium.

The interferon-like inhibitor was produced in large amounts from the 2nd to 7th day-incubation but disappeared by the 15th day.

4 Effect of length of pretreatment of DKC on the interfering activity of the interferon-like inhibitor

DKC were pretreated with the test materials for various periods of time prior to the challenge with ICH virus.

The interfering phenomenon was again observed only in the DKC pretreated with culture fluids of normal HeLa cells (tab. 4).

TABLE 4 *Effect of length of pretreatment of DKC on the interfering activity of the interferon-like inhibitor*

TEST MATERIAL	LENGTH OF PRETREATMENT IN HOURS*1						
	0	1.5	4	8	16	24	48*2
Fluids of normal HeLa cells	5.8	5.5	4.8	3.2	3.5	3.8	5.5
Fluids of ICH virus infected HeLa cells	6.2	6.2	5.5	5.5	5.8	5.8	5.8
Medium control	6.2	5.8	5.8	5.2	6.2	6.2	5.5

*1 Length of pretreatment of DKC with the test materials before the challenge of ICH virus

*2 Following the 24 hr pretreatment, the test materials were removed and washed out from DKC. The cells were then cultivated for another 24 hr with fresh medium before virus challenge.

From the table, it could be said that 8 hr or more pretreatment of DKC with culture fluids of normal HeLa cells was necessary to establish the interfering activity.

When DKC, pretreated with interferon-like inhibitor for 24 hr was washed and cultivated

with a fresh maintenance medium for another 24 hr before virus challenge, no interfering activity was observed. The data indicated that the interfering activity of the interferon-like inhibitor was no longer found at 24 hr after removal of the inhibitor from the DKC.

5 Effect of concentration of the interferon-like inhibitor on the interfering activity

In the preceding experiments, 10% culture fluids in the maintenance medium were used as the test material. Effect of concentration of the interferon-like inhibitor on the interfering activity was studied. The materials with various concentrations of culture fluids as shown in table 5 were tested.

TABLE 5 *Effect of concentration of interferon-like inhibitor on the interfering activity*

TEST MATERIAL	CONCENTRATION OF TEST MATERIAL IN PERCENTAGES						
	100	50	25	10	5	2	0
Fluids of normal HeLa cells	3.5	3.5	3.8	3.5	4.2	4.8	5.8
Medium control	6.2	5.8	6.2	6.2	5.5	5.8	6.2

From the data in the table, 10% contents of the culture fluids of normal HeLa cells in the medium were enough to give maximum interfering activity.

6 Effect of anti-ICH virus immune serum on interfering activity of the interferon-like inhibitor

Whether the interferon-like inhibitor has any antigenic relation with the ICH virus was

TABLE 6 *Effect of anti-ICH virus immune serum on interfering activity of the interferon-like inhibitor*

TEST MATERIAL	EXP. 1	EXP. 2
Fluids of normal HeLa cells		
plus immune serum	3.8	4.2
plus normal serum	3.8	3.8
Fluids of ICH virus infected HeLa cells		
plus immune serum	5.5	6.2
plus normal serum	6.2	5.8
Medium control		
plus immune serum	5.2	6.2
plus normal serum	5.8	6.2

Anti-ICH virus immune (neutralizing antibody titer of 1 : 4096) or normal guinea-pig serum was diluted to 1 : 50 and was added to an equal volume of 20% culture fluids. The mixture was incubated for 1 hr at 37°C, and served as the test material.

tested, using anti-ICH virus immune guinea-pig serum.

The serum, neutralizing antibody titer of 1 : 4096, was diluted to 1 : 50 and was added to an equal volume of 20% culture fluids of normal HeLa cells. The mixture was incubated 1 hr at 37°C and the interfering activity was tested. As control, normal guinea-pig serum treated likewise was used.

The interfering activity of the culture fluids of normal HeLa cells was not influenced by immune serum (tab. 6).

7 Interfering activity of culture fluids of HeLa cells against other viruses

Whether the interferon-like inhibitor is effective against other virus multiplications in DKC was tested. Human adenovirus type 7, Western equine encephalomyelitis virus (WEEV) and Newcastle disease virus (NDV) were used for this purpose.

The data obtained are shown in table 7. A slight interfering activity against adenovirus type 7 was observed in 2 experiments performed. But neither WEEV nor NDV were showed any CPE or multiplication in DKC even in the control system, so that we could not confirm interfering activity of the interferon-like inhibitor against these viruses.

TABLE 7 *Interfering activity of culture fluids of HeLa cells against other viruses*

TEST MATERIAL	CHALLENGED VIRUS*1				
	Adeno V		WEEV	NDV	ICHV
	Exp. 1	Exp. 2			
Fluids of normal HeLa cells	4.2	3.8	—*2	—	3.8
Fluids of ICH virus infected HeLa cells	5.5	5.2	—	—	6.2
Medium control	5.8	5.2	—	—	5.8

*1 Adeno V : Human adenovirus type 7
WEEV : Western equine encephalomyelitis virus
NDV : Newcastle disease virus
ICHV : Infectious canine hepatitis virus
*2 No sign of virus multiplication was observed.

8 Effect of physical and chemical treatments on the interferon-like inhibitor

Treatment of the interferon-like inhibitor with various agents was performed and the data obtained were summarized in table 8.

Dialysis against a pH 2 solution and heating at 58°C for 30 min had no effect on the activity of the interferon-like inhibitor. DNase or RNase had no effect. However, its activity almost ceased after incubation with trypsin.

9 Presence of the interferon-like inhibitor in the culture fluids of 2 other lines of HeLa cells

In the above experiments, only A-HeLa cells were used. To confirm whether the

TABLE 8 *Effect of various treatments on the interferon-like inhibitor*

TEST MATERIAL*1	FLUIDS OF NORMAL HELA CELLS		MEDIUM CONTROL
	Treated	Untreated	
Dialysis pH 2	3.5*2	3.8	6.2
Heated 58°C, 30 min	4.2	3.9	6.0
Trypsin	5.7	3.7	6.2
DNase	3.7	3.7	6.4
RNase	4.0	4.1	5.9

*1 Methods of treatment with various agents were described in MATERIALS AND METHODS

*2 Mean titer of 2 experimental data

interferon-like inhibitor was present in normal fluids of other lines of HeLa cells, B-HeLa cells and HeLa-S3, a clonal subline of HeLa cells, were used.

As shown in table 9, the culture fluids of these HeLa cells showed similar interfering activity as A-HeLa cells.

TABLE 9 *Presence of interferon-like inhibitor in culture fluids of 3 lines of normal HeLa cells*

TEST MATERIAL*1	EXP. NO.		
	1	2	3
A-HeLa	3.8	3.5	4.2
B-HeLa	3.5	3.5	3.8
HeLa-S3	3.8	ND*2	4.8
Medium control	5.5	6.2	5.8

*1 Normal culture fluids of the following 3 lines of HeLa cells were used.
A-HeLa : HeLa cells maintained in the authors' laboratory
B-HeLa : HeLa cells maintained in the Hokkaido Institute of Public Health
HeLa-S3 : Clonal subline of HeLa cells maintained in the same laboratory with that of B-HeLa.

*2 Not done

10 Possible role of contamination of PPLO in the HeLa cells

It was suggested that PPLO can promote production of interferon in cultured cells. Therefore, contamination of PPLO was tested in A-HeLa cells used.

The fluid and cellular fractions of the HeLa cells were inoculated into Bacto PPLO broth (Difco) containing 10% calf serum. Following 72 hr cultivation at 37°C, they were plated on Bacto PPLO agar (Difco) containing 5% calf serum. After 72 hr incubation at 37°C, the appearance of PPLO colony was examined. No PPLO was detected from A-HeLa cells.

Examination by electron microscopy of PPLO in concentrated culture fluids of A-HeLa cells also proved negative.

DISCUSSION

The present authors reported that HeLa cells were capable of supporting partial multiplication of the ICH virus[9] and also that HeLa cells infected with the ICH virus showed a persistent low grade infection[10]. We assumed a participation of interferon in this virus-cell interaction. HO & ENDERS demonstrated that the initiation and maintenance of persistent infection with poliovirus in human amnion cell cultures was partially dependent upon production of interferon. CHANY also postulated that interferon contributed to the establishment of para-influenza carrier cultures.

Thereupon we studied to determine whether ICH virus induce interferon in HeLa cells and whether interferon is related to the maintenance of persistent infection of ICH virus in HeLa cells.

In a preliminary experiment, we failed to demonstrate interferon production in HeLa cells infected with ICH virus when tested in the same cell system.

Hitherto, the action of interferon has been thought to be cell specific, except that of tumor cells.

HO & ENDERS noted that the fluids of HeLa cell cultures persistently infected with poliovirus contained interferon-like activity that could be observed when tested in primary human-amnion or kidney cell cultures, but not in uninfected HeLa cells. CHANY also found that interferon produced by the parainfluenza virus in KB cells was not very effective in KB cells but could be better demonstrated in primary human-amnion cell cultures. Taking these results into consideration, we attempted to demonstrate the interferon produced by ICH virus in HeLa cells, using DKC.

As described in the text, the results obtained were opposite to our expectation. The interferon was not demonstrated in infected HeLa cells but a similar inhibitor was found in normal HeLa cells.

As to physicochemical natures of the interferon-like inhibitor, the inhibitor was not sedimented by centrifugation at 36,000 rpm for 90 min and was also shown to be non-dialyzable against a pH 2 solution. Its activity was not inactivated by heating at 58°C for 30 min or by treatment with both DNase and RNase. But the activity of the inhibitor was largely lost after treatment with trypsin. These properties of the interferon-like inhibitor was similar to those of interferon (HO, 1962). However, origins of the materials were quite different, namely the interferon was produced in cell cultures after infection of viruses, but the interferon-like inhibitor described here was produced in uninfected, normal HeLa

cell cultures.

Interferon had been originally defined as a soluble factor produced in cells in vitro or in vivo by viruses alone. But later, the definition has been extended to imply similar factors such as produced by non-viral foreign nucleic acid[15], by a certain bacteria, for example, Brucella[5,21], by bacterial endotoxin[7], and by antibiotics, for example, statolon[11] and helenine[17]. YERSHOV & ZHDANOV reported the possibility that PPLO enhance the production of interferon.

Therefore, many factors other than virus can induce interferon, and all cells may have potential activity to produce interferon. But the reason why the present inhibitor was produced in normal HeLa cells is obscure. All factors relating to the cultivation of HeLa cells should be examined.

YERSHOV & ZHDANOV reported that PPLO alone did not induce interferon production in chick embryo fibroblasts, but preinfection with PPLO increased the production of interferon in the same cells which were infected with Eastern equine encephalomyelitis virus and Venezuelan equine encephalomyelitis virus.

OGATA et al. found that 40 out of 48 (83%) cell-samples of continuous cell lines tested were contaminated with PPLO. The present authors tried to isolate PPLO from the HeLa cells used but the result was negative. The methods used for isolation of PPLO may be incomplete. Therefore, we could not negate the possibility of the participation of PPLO in the present experiment.

No viral multiplication was observed when HeLa cells were inoculated with small doses of ICH virus such as less than 3.5 log $TCID_{50}$ per ml. For the multiplication of ICH virus in HeLa cells, larger inoculum such as more than 4.5 log $TCID_{50}$ was necessary (KINJO & YANAGAWA, 1967[9]).

Therefore, the reason why the interferon-like inhibitor was not demonstrated in HeLa cells infected with ICH virus is assumed to be because the inhibitor was neutralized by ICH virus.

It is probable that, after being cultivated for many years under different conditions, the lines of HeLa cells are not identical. In fact, differences between HeLa cell lines have been described with respect to the resistance to viruses[19], the requirement of serum[16], mode of growth[14] carbohydrate metabolism[3] and chromosome numbers[18]. From the above viewpoints, we compared 2 different lines of HeLa cells and HeLa-S3, a clonal subline of HeLa cells. But no difference was observed between the 3 lines of HeLa cells.

Recently, interferon-like inhibitors have been reported to be produced in normal cells. AKIHAMA et al. reported that an interferon-like viral inhibitor was present in fluids of normal HeLa, Hep-2, FL and KB cells. The inhibitor was not inactivated by heating at 56°C for 30 min and 100°Cfor 10 min, by treatments with ether, DNase, RNase and also with trypsin. These results were similar to

that of the authors' interferon-like inhibitor, except the sensitivity to trypsin. NAGANO et al. found that phagocytes derived from rabbit peritoneal cavity could be produced interferon-like factor after the phagocytes were suspended in PBS or physiological saline and leaved at 37°C.

From the above findings and our results, it could be said that normal HeLa cells can produce an interferon-like inhibitor, under usual conditions. Such possibility should be taken into consideration when virus-cell interactions are studied.

SUMMARY

Normal HeLa cells produced an interferon-like inhibitor against ICH virus and this was demonstrated in cultures of normal HeLa cells when tested in DKC. No interferon was detected in cultures of HeLa cells infected with ICH virus.

This interferon-like inhibitor was not sedimented by centrifugation at 36,000 rpm for 90 min and was shown to be non-dialyzable against a pH 2 solution. Its activity was not inactivated by heating at 58°C for 30 min or by treatment with anti-ICH virus immune serum. Neither DNase nor RNase had any effect on the activity of the inhibitor. However, its activity was largely lost after treatment with trypsin.

These properties of the interferon-like inhibitor were similar to those of interferon. However the origin of the interferon-like inhibitor was completely distinct from that of interferon because the former was produced in the cultures of normal HeLa cells.

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