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1. Shigeshi Toyoshima, Takeo Ueda, and Yoshiko Seto: The Accelerating Effect of Arginine on Cytophatic Effect of Adeno Virus.

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A long incubation period required for adeno virus to show a cytophatic effect has been discouragement for both the screening test of anti-adeno compound and for the prospect of adapting plaque technique for this virus. Anti-adeno compounds could not be screened effectively in such a long incubation period, and host cells could not be kept alive under an agar overlay, a condition which is most essential for plaque assay. To overcome this problem, the incubation time must be shortened. For this purpose, Schlesinger and Bonifas[1] reported that arginine rich Eagle's medium accelerated the appearance of cytophatic effect of adeno virus type 2 strain in KB cells and that the system of adeno virus-KB cells could be promising for plaque assay of the virus.

To improve the screening method of anti-adeno compounds, an investigation was made on the accelerating effect of arginine on the appearance of adeno virus CPE in various cell lines. Among the cell lines, Hep. No. 2 cells exhibited the fastest appearance of the cytophatic effect when arginine-rich YLA medium was employed.

This report describes the adaptation of arginine-rich YLA medium-Hep. No. 2 cells system to the screening test of anti-adeno compounds.

Experimental

Materials and Methods

1) Host cells: The following host cells were employed in the experiment; HeLa wild type cells, HeLa-S_3 cloning strain cells, FL cells and Hep. No. 2 cells.

2) Viral materials: Type 2 strain of adeno virus was used. Prior to experiments, several passages through each cell line were carried out. After complete degeneration of cells, the mixture of culture fluid and cells was frozen and thawed five times, and then was centrifuged at 3000 r.p.m. for 15 min. The supernatant was removed and stored at −20°.

3) Media: For growth medium, 20% bovine serum supplemented YLA medium was used. And for maintenance medium, both of 5% horse serum supplemented YLA medium and 15% horse serum supplemented Eagle's medium were used.

4) General procedures: The monolayer of each cell line used was prepared by inoculating 2×10^5 cells in 1 cc. per tube. After these tubes were incubated at 37° for 4 days, the monolayer cell sheet was established, these tubes were washed three times with phosphate buffered saline (pH. 7.6). 0.1 cc. of the dilution of a viral material and 0.9 cc. of the maintenance medium were added, and then the whole mixture was incubated at 37°. From daily microscopic observations, the first day of appearance of the cytophatic effect was recorded. For calculation of tissue culture infective dosis fifty,[*2] Reed and Muench's method[2] was employed. Other experimental details will be described in respective sections.

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*2 Abbreviation. $TCID_{50}$.

1) R.W. Schlesinger, V. Bonifas: Federation Proc. 18, 2196 (1959).

2) L.J. Reed, H. Muench: Am. J. Hyg. 27, 493 (1938).

Results

1) Toxicity of L-Arginine Hydrochloride to Each Cell Line

First of all, the toxicity of L-arginine hydrochloride to each cell line was determined. Arginine containing YLA medium and Eagle's medium were prepared by adding L-arginine hydrochloride at the concentrations of 30, 45, 60, 75, 120, and 150 γ/cc. Aliquots of each of 1 cc. of these arginine containing media was added into tubes, in which the monolayer of each of the used cell lines had been established, and then these tubes were incubated at 37° for 7 days. From daily microscopic observation, the maximum nontoxic concentration of arginine on each cell line was determined. The toxicity of arginine in Eagle's medium was found stronger than that in YLA medium against all of the used cell lines, that is, the YLA medium which contained 150 γ/cc. of arginine did not show any toxic effect on each cell line while Eagle's medium containing 30 γ/cc. of arginine showed an apparent cell degeneration 5 or 6 days after the addition of the medium into tubes. Based on this result, the arginine containing YLA medium was employed throughout the subsequent experiments.

2) Accelerating Effect of Arginine on the Cytophatic Effect of Adeno Virus in Cell Lines

The cytophatic effect of adeno virus in the arginine-rich YLA medium was examined by using various cell lines.

After the monolayer cell sheet of each of used cell lines was established, the cells were washed three times with phosphate buffered saline, and then 0.9 cc. of the YLA medium containing 150 γ/cc. of arginine and 0.1 cc. of 10^{-2} dilution of adeno type 2 strain virus (the $TCID_{50}$ of this strain was $10^{-6.5}$ from the observation for 21 days.) were added into these tubes and incubated at 37° for 10 days. From daily microscopic observation, the first day of appearance of the cytophatic effect was recorded.

Table I. Accelerating Effect of Arginine on Cytophatic Effect of Adeno Type 2 Virus

Used cell line		HeLa (wild)		HeLa (S_3)		FL		Hep. No. 2	
		C[a]	T[b]	C	T	C	T	C	T
Results	A[c]	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	B[d]	6, 6, 6.	6, 6, 6.	6, 7, 6.	6, 6, 7.	6, 6, 6.	6, 6, 6.	6, 7, 7.	4, 4, 4.

a) Control (YLA medium without arginine)
b) Treated group (YLA medium containing 150 γ/cc. of arginine)
c) Numbers of tubes with positive cytophatic effect/Number of total tubes
d) The day of the appearance of cytophatic effect

From Table I, it can be seen that the appearance of the cytophatic effect in Hep. No. 2 cells was accelerated clearly by the addition of 150 γ/cc. of arginine, while in other cell lines such as that of HeLa-wild type, HeLa-S_3 strain and FL cells, no accelerating effect of arginine was found.

3) Minimal Effective Concentration of Arginine for the Acceleration of the Appearance of Cytophatic Effect of Adeno Virus

On the basis of above findings, the minimal effective dose of arginine to accelerate the appearance of the cytophatic effect of adeno virus in Hep. No. 2 cells was investigated. Aliquots of 0.9 cc. of the YLA medium containing 30, 45, 60, 75, 120, and 150 γ/cc. of arginine and 0.1 cc. of 10^{-2} dilution of adeno type 2 strain ($TCID_{50}$ was $10^{-6.5}$) were added into tubes which contained the established monolayer cell sheet, then these tubes were incubated at 37° for 10 days. From the daily microscopic observation, the first day of the appearance of the cytophatic effect was recorded.

Table II. Minimal Effective Concentration of Arginine to Accelerate Cytophatic Effect of Adeno Type 2 Virus in Hep. No. 2 Cells

Amounts of arginine in YLA medium (γ/cc.)		0	30	45	60	75	120	150
Results	A[a]	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	B[b]	6, 7, 7.	5, 6, 6.	6, 6, 7.	5, 5, 6.	5, 5, 6.	5, 5, 5.	4, 4, 4.

a) Numbers of tubes with positive cytophatic effect/Number of total tubes
b) The day of the appearance of cytophatic effect of adeno type 2 virus

Table II indicates that the minimal effective concentration of arginine to accelerate the cytophatic effect was 75 γ/cc. Below this level, the effect of arginine was not well defined.

4) Prompt Increase of Tissue Culture Dosis Fifty of Adeno Virus by the Addition of Arginine in Hep. No. 2 Cells

According to above findings, it may be said that $TCID_{50}$ of adeno virus should increase promptly and the incubation period could be shortened by using the arginine-rich YLA-Hep. No. 2 cells system. To make this point clear, the following experiments were carried out.

Aliquots of 0.9 cc. of the YLA medium containing 150 γ/cc. of arginine and 0.1 cc. of the 10 fold dilutions of adeno type 2 strain were added into tubes in which the monolayer of Hep. No. 2 cells had beed established, and then these tubes were incubated at 37° for 14 days. From the daily microscopic observation, the daily $TCID_{50}$ of both the control and the arginine rich group was calculated by using Reed and Muench's method.[2]

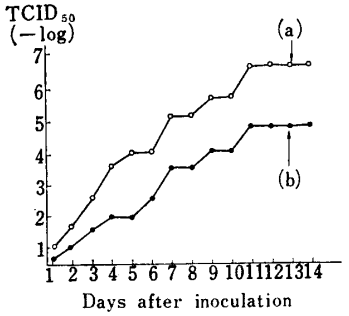

Fig. 1. Prompt Increase of $TCID_{50}$ of Adeno Type 2 Virus by using the Maintenance Medium supplemented with Arginine and Hep. No. 2 Cells

YLA medium supplemented with 150 γ/cc. of arginine and 5% horse serum
(a) The treated group in which arginine was added to YLA medium
(b) The control group in which no arginine was added to YLA medium

Fig. 1 shows that the $TCID_{50}$ of the culture using ordinary YLA medium is $10^{-4.5}$ 14 days after the inoculation while that of the culture using the arginine-rich YLA medium was $10^{-5.5}$ even in 7 days after adding viral dilutions. These results suggest that the prompt increase of $TCID_{50}$ of adeno virus should be obtained by using the arginine-rich medium in Hep. No. 2 cells.

5) Effect of 3-(Alkoxyphenoxy)-1,2-propandiols on Adeno Virus in Both YLA and Arginine-rich YLA Medium

On the basis of above findings, it was suggested that the culture using the arginine-rich medium was more useful than that using ordinary YLA medium to shorten the incubation period in screening for anti-adeno compounds. The anti-adeno effect of 3-(alkoxyphenoxy)-1,2-propandiols was described by Ueda, Toyoshima, Takahashi, and Muraoka.[3] In their experiment, the ordinary YLA medium supplemented with 5% horse serum was employed as maintenance medium. Thus, it remained for the present

3) T. Ueda, S. Toyoshima, K. Takahashi, M. Muraoka : This Bulletin. 8, 921 (1960).

authors to find if the arginine-rich medium actually is more useful than the ordinary YLA medium to shorten the incubation time for the screening test.

For this purpose, 3-(alkoxyphenoxy)-1,2-propandiol compounds were employed as representatives of anti-adeno agents. 3-(*o*-Hexyloxyphenoxy)-, 3-(*o*-octyloxyphenoxy)-, and 3-(*p*-dodecylphenoxy)-1,2-propandiols were effective on type 1 strain of adeno virus in a previous study.[3]

After a monolayer of Hep. No. 2 cells was established, the growth medium was removed and the cell sheet was washed with phosphate buffered saline (pH. 7.6). In one group using arginine-rich medium, 0.8 cc. of the arginine-rich YLA medium added with 5% horse serum and 0.1 cc. of a test compound solution were introduced into tubes, and then, in minutes, 0.1 cc. of type 2 strain dilution of adeno virus was used to inoculate. In other group using the ordinary medium, the YLA medium supplemented with 5% horse serum but without arginine was employed. For control, phosphate buffered saline solution was employed instead of a test compound solution. These tubes were incubated at 37° for 21 days, and $TCID_{50}$ of each of the controls and the treated groups was calculated.

Table III. Effect of 3-(Alkoxyphenoxy)-1,2-propandiols on Adeno Type 2 Virus in Hep. No. 2 Cells

Compounds	Concn. of drugs ($10^{-x}M$)	Medium	$TCID_{50}$ in number of days after inoculation (−log)										
			1	3	5	7	9	11	13	15	17	19	21
None			<1	1.5	2	3.5	4	5	5	5.5	6	6.5	6.5
O—6[a]	4	A[d]	<1	1	1.5	3.5	4	5	5	5.5	6	6.5	6.5
O—8[b]	4		<1	<1	1	3	4	5	5	5.5	6.5	6.5	6.5
P-12[c]	4		<1	<1	1	3	4	5	5	5	6	6.5	6.5
None			1	2.5	4	5.5	6	6	6.5	6.5	6.5	6.5	6.5
O—6	5	B[e]	<1	1.5	4	5.5	6	6	6.5	6.5	6.5	6.5	6.5
O—8	4		<1	1.5	4	5	6	6	6.5	6.5	6.5	6.5	6.5
P-12	4		<1	1	3.5	5	6	6	6.5	6.5	6.5	6.5	6.5

a) 3-(*o*-hexyloxyphenoxy)-1,2-propandiol
b) 3-(*o*-octyloxyphenoxy)-1,2-propandiol
c) 3-(*p*-dodecyloxyphenoxy)-1,2-propandiol
d) YLA medium supplemented with 5% horse serum, but without addition of arginine.
e) YLA medium supplemented with 5% horse serum and 150 γ/cc. of arginine.

As shown in Table III, $TCID_{50}$ of the treated groups of both types was lower than that of the control several days after viral inoculation. The difference between $TCID_{50}$ of the treated and the control of ordinary maintenance culture could not be found 6 days after the inoculation. The maximal $TCID_{50}$, however, was obtained after 18 days, while in the culture with the arginine-rich maintenance medium, the difference between $TCID_{50}$ of the control and the treated groups disappeared 3 days after inoculation; the maximal $TCID_{50}$ of both of them was obtained after 12 days.

These results indicate that the use of the arginine-rich maintenance medium is useful in shortening the incubation time in the screening test of anti-adeno compounds.

Discussion

The experiments reported here demonstrates that the addition of arginine in YLA medium accelerated the appearance of cytophatic effect of adeno virus in Hep. No. 2 cells, and as a result, a prompt increase of $TCID_{50}$ of the virus was obtained. At present, no explanation has not been given for the long incubation time needed to show the cytophatic effect of adeno virus. From the findings reported by Kjellén,[4]

4) L. Kjellén: Arch. ges. Virusforsch. **7**, 110 (1956).

Green,[5] and Pereira,[6] two possibilities were suggested, one of which was that the newly formed virus associated tightly with cells and were not released into the fluid phase of a culture until later, thus the virus could hardly infect the surrounding cells. Another one is that the cytophatic effect might be caused by some of the toxic substances produced by the virus in the process of replication, and the delayed cytophatic effect in the infection of adeno virus suggests that such toxic substances were poorly produced. On the basis of inadequate findings on the mechanism of cytophatic effect of adeno virus, it was difficult at present to offer any concrete explanation as to the effect of arginine on cytophatic effect of adeno virus in both KB[1] and Hep. No. 2 cells. Further inquiry into this explains the usual delayed appearance of cytophatic effect. Present results showed that the long incubation time required for adeno virus to obtain cytophatic effect could be shortened by using an arginine-rich medium, and that the use would be highly beneficial in the screening test of anti-adeno compounds.

Summary

The accelerating effect of arginine on cytophatic effect of adeno virus was investigated by using such cell lines as HeLa wild type, HeLa S_3 strain, FL and Hep. No. 2 cells. The effect of arginine in promoting a cytophatic effect was demonstrated in the Hep. No. 2 cells-arginine-rich maintenance medium system. In the investigation on the effect of 3-(alkoxyphenoxy)-1,2-propandiols on adeno virus, the use of the Hep. No. 2 cells-arginine-rich maintenance medium system was found to be most promising for the screening test of anti-adeno compounds.

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5) M. Green : Virology, **13**, 169 (1961).
6) H. Pereira, A.C. Allison : *Ibid.*, **7**, 300 (1959).

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2. Shigeshi Toyoshima, Takeo Ueda, Tadakazu Tsuji, Yoshiko Seto, and Junko Nomoto : Inhibitory Effect of Guanidine on Several Viruses Including Polio and Measles.

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In the preceding report,[1] it was described by Ueda and Toyoshima that 2-imino-5-methylhexahydro-*s*-triazine exerted an inhibitory effect on the growth of polio virus in HeLa cells. However, from the further research it was inferred that the effect of this compound should not be due to its own property, but should be ascribed to an action of one of decomposed substances from the above compound, guanidine. According to this inference, this work was prompted by the present authors to investigate.

As to the antiviral effect of guanidine, Bawden and Pirie[2] reported that it showed a virus inactivating effect on tabacco mosaic virus only at a high concentration of 2.7*M*.

*1 35, Shinanomachi, Shinjuku-ku, Tokyo (豊島 滋, 上田武雄, 辻 忠和, 瀬戸淑子, 野本純子).
1) T. Ueda, S. Toyoshima : Nippon Yakurigaku Zasshi, **56**, 145 § (1960).
2) F.C. Bawden, N.W. Pirie : Biochem. J., **34**, 1278 (1940).