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A long incubation period required for adeno virus to show a cytopathic effect has been discouragement for both the screening test of anti-adoeno compound and for the prospect of adapting plaque technique for this virus. Anti-adoeno 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 reported that arginine rich Eagle's medium accelerated the appearance of cytopathic 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-adoeno 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 cytopathic 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-adoeno compounds.

**Experimental**

*Materials and Methods*

1) Host cells: The following host cells were employed in the experiment; HeLa wild type cells, HeLa-S, 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°C.

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 \(2 \times 10^5\) cells in 1 cc. per tube. After these tubes were incubated at 37°C 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°C. From daily microscopic observations, the first day of appearance of the cytopathic effect was recorded. For calculation of tissue culture infective dose fifty,* Reed and Muench's method** was employed. Other experimental details will be described in respective sections.

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* Abbreviation. TCID_{50}.
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 Cytopathic Effect of Adeno Virus in Cell Lines

The cytopathic 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 TCID₅₀ of this strain was 10^-4.5 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 cytopathic effect was recorded.

| Table I. Accelerating Effect of Arginine on Cytopathic Effect of Adeno Type 2 Virus |
|--------------------------------|----------------------------|-------------------|------------------|----------------|-----------------|------------------|
| Used cell line | HeLa (wild) | HeLa (S₅) | FL | Hep. No. 2 |
|----------------|-------------|-----------|----|-----------|----------------|------------------|
|                | C(a)        | T(b)      | C  | T         | C              | T                |
| Results        | 3/3         | 3/3       | 3/3| 3/3       | 3/3            | 3/3              |
| a) Control (YLA medium without arginine) | b) Treated group (YLA medium containing 150 γ/cc. of arginine) | c) Numbers of tubes with positive cytopathic effect/Number of total tubes | d) The day of the appearance of cytopathic effect |

From Table I, it can be seen that the appearance of the cytopathic 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-S₅ strain and FL cells, no accelerating effect of arginine was found.

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

On the basis of above findings, the minimal effective dose of arginine to accelerate the appearance of the cytopathic 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 (TCID₅₀ was 10^-4.5) 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 cytopathic effect was recorded.
Table II. Minimal Effective Concentration of Arginine to Accelerate Cytopathic Effect of Adeno Type 2 Virus in Hep. No. 2 Cells

<table>
<thead>
<tr>
<th>Amounts of arginine in YLA medium (γ/cc.)</th>
<th>0</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>120</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>6, 7, 7. 5, 6, 6, 6, 6, 7. 5, 5, 5, 5, 6, 5, 5, 5, 4, 4, 4.</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup> Numbers of tubes with positive cytopathic effect/Number of total tubes

<sup>b</sup> The day of the appearance of cytopathic effect of adeno type 2 virus

Table II indicates that the minimal effective concentration of arginine to accelerate the cytopathic effect was 75γ/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<sub>50</sub> 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 150γ/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 been established, and then these tubes were incubated at 37° for 14 days. From the daily microscopic observation, the daily TCID<sub>50</sub> of both the control and the arginine rich group was calculated by using Reed and Muench's method.<sup>3</sup>

![Fig. 1. Prompt Increase of TCID<sub>50</sub> of Adeno Type 2 Virus by using the Maintenance Medium supplemented with Arginine and Hep. No. 2 Cells](image)

Fig. 1 shows that the TCID<sub>50</sub> of the culture using ordinary YLA medium is 10<sup>-4.5</sup> 14 days after the inoculation while that of the culture using the arginine-rich YLA medium was 10<sup>-3.1</sup> even in 7 days after adding viral dilutions. These results suggest that the prompt increase of TCID<sub>50</sub> of adeno virus should be obtained by using the arginine-rich medium in Hep. No. 2 cells.

5) Effect of 3-(Alk oxyphenox y)-1,2-propan diols 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-(alkoxyphenox y)-1,2-propan diols was described by Ueda, Toyoshima, Takahashi, and Muraoka.<sup>3</sup> In their experiment, the ordinary YLA medium supplemented with 5% horse serum was employed as maintenance medium. Thus, it remained for the present

<sup>3</sup> 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-adenovirus agents. 3-(o-Hexyloxyphenoxy)-, 3-(o-octyloxyphenoxy)-, and 3-(p-dodecyloxyphenoxy)-1,2-propandiol were effective on Type 1 strain of adenovirus in a previous study.\(^4\)

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 adenovirus 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°C for 21 days, and TCID\(_{50}\) of each of the controls and the treated groups was calculated.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Conc. of drugs (10(^{-2})M)</th>
<th>Medium</th>
<th>TCID(_{50}) in number of days after inoculation (−log)</th>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>1 3 5 7 9 11 13 15 17 19 21</td>
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<tr>
<td>O−6(^a)</td>
<td>4</td>
<td>A(^d)</td>
<td>&lt;1 1.5 3.5 4 5 5 5.5 6 6.5 6.5</td>
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<tr>
<td>O−8(^b)</td>
<td>4</td>
<td></td>
<td>&lt;1 &lt;1 1 3 4 5 5 5.5 6 6.5 6.5</td>
</tr>
<tr>
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<td>&lt;1 &lt;1 &lt;1 1 3 4 5 5 5 6 6.5 6.5</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>B(^e)</td>
<td>1 2.5 4 5.5 6 6 6.5 6.5 6.5 6.5</td>
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<tr>
<td>O−6</td>
<td>5</td>
<td></td>
<td>&lt;1 1.5 4 5.5 6 6 6.5 6.5 6.5 6.5</td>
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<tr>
<td>O−8</td>
<td>4</td>
<td></td>
<td>&lt;1 1.5 4 5 6 6 6.5 6.5 6.5 6.5</td>
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<tr>
<td>P−12</td>
<td>4</td>
<td></td>
<td>&lt;1 1 3.5 5 6 6 6.5 6.5 6.5 6.5</td>
</tr>
</tbody>
</table>

\(^a\) 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-adenovirus compounds.

**Discussion**

The experiments reported here demonstrates that the addition of arginine in YLA medium accelerated the appearance of cytopathic effect of adenovirus 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 cytopathic effect of adenovirus. From the findings reported by Kjellén,\(^4\)

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 cytopathic effect might be caused by some of the toxic substances produced by the virus in the process of replication, and the delayed cytopathic effect in the infection of adenovirus suggests that such toxic substances were poorly produced. On the basis of inadequate findings on the mechanism of cytopathic effect of adenovirus, it was difficult at present to offer any concrete explanation as to the effect of arginine on cytopathic effect of adenovirus in both KB13 and Hep. No. 2 cells. Further inquiry into this explains the usual delayed appearance of cytopathic effect. Present results showed that the long incubation time required for adenovirus to obtain cytopathic 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 cytopathic effect of adenovirus was investigated by using such cell lines as HeLa wild type, HeLa S1 strain, FL and Hep. No. 2 cells. The effect of arginine in promoting a cytopathic effect was demonstrated in the Hep. No. 2 cells-arginine-rich maintenance medium system. In the investigation on the effect of 3-(alkoxyphenoxyl)-1,2-propanediols on adenovirus, 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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In the preceding report,1) it was described by Ueda and Toyoshima that 2-imino-5-methylhexahydro-5-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 Pirie5) reported that it showed a virus inactivating effect on tobacco mosaic virus only at a high concentration of 2.7M.

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