Enhancement of Adenovirus Plaque Formation on HeLa Cells by Magnesium Chloride

(Received 9 September 1970)

The effect of divalent cations on the susceptibility of mammalian cells to infection by viruses has been the subject of several investigations. Calcium enhances poliovirus plaque formation on rabbit embryo kidney cells (Cooper, 1961) and magnesium increases the susceptibility of monkey kidney cells to poliovirus (Wallis & Melnick, 1962). Plaque formation by certain rhinoviruses in HeLa cells is enhanced by higher concentrations of calcium and magnesium in the overlay medium (Fiala & Kenny, 1966; Stott & Tyrrell, 1968). The increased susceptibility of monkey kidney cells to poliovirus results from the earlier release of virus from infected cells (Wallis & Melnick, 1962), and the release of rhinovirus from HeLa cells is also greatly enhanced in the presence of high levels of MgCl₂ (Fiala & Kenny, 1967). This communication provides evidence that MgCl₂ also enhances plaque formation by human adenoviruses on HeLa cell monolayers and that the effect is due to an increase in the rate of virus release from infected cells.

The adenoviruses were grown and plaque-assayed on HeLa cells maintained in modified Eagle's medium (twice the normal concentration of amino acids and vitamins) supplemented with 10% calf serum, and grown at 37 ° in rotating Winchester bottles. Adenovirus stocks were prepared from HeLa cell monolayers grown thus and infected at a multiplicity of 1 p.f.u./cell. Infected cells were maintained at 37 ° in Eagle's medium containing 10% foetal calf serum and 2 days after infection the cells were removed from the surface by shaking or with trypsin, washed twice in tris saline, resuspended in tris saline, and frozen and thawed three times to release virus. The cell extract was centrifuged at 2000 rev./min for 15 min. and the supernatant fluid retained and stored at −20 °.

Adenovirus was assayed by infecting confluent monolayers of HeLa cells in 50 mm. plastic Petri dishes (a/s NUNC, Denmark) with 0.1 ml. volumes of virus diluted in buffered saline. Virus was adsorbed at 37 ° for 90 min., then monolayers were overlaid with 5 ml. of 0.65% Noble agar (Difco) in Eagle's medium containing 2% foetal calf serum (Flow Lab. Ltd) and incubated at 37 ° in humidified air containing 5% CO₂. After incubation for 5 days an additional 2 ml. of agar overlay medium was added to the cultures, and on the 6th or 7th day after infection a further 2 ml. of overlay containing neutral red was added. In many of the experiments 25 mM-MgCl₂ was incorporated in the overlay medium: normal Eagle's medium contains 0.8 mM-MgSO₄·7H₂O and 1.8 mM-CaCl₂, without MgCl₂.

Initial experiments were carried out using adenovirus type 5 and a supplementary concentration of 25 mM-MgCl₂. Under the standard conditions used here for titration of adenovirus type 5 on HeLa cells, plaques do not appear until 7 to 8 days after infection and the maximum plaque count is not attained until 10 to 12 days. Plaques appeared 2 to 3 days earlier, and maximum plaque counts were obtained 2 to 3 days earlier in cultures supplemented with 24 mM-MgCl₂. To determine the optimum conditions for plaque enhancement, MgCl₂ concentrations from 12 to 50 mM were tested: the results of one such experiment are shown in Table 1. A less pronounced effect was given by 12 mM-MgCl₂ than by 25 mM-MgCl₂, and no further improvement was observed above 25 mM. Concentrations of 45 and 50 mM gave a 25% lower count, and plaques, though large, were less clear than at 25 mM.
However, even after a week in the presence of 50 mM-MgCl₂, HeLa cells remained viable, as shown by their ability to take up neutral red. A concentration of 25 mM-MgCl₂ was therefore optimal for use in the improved plaque assay of adenovirus type 5. The sizes of the plaques produced in the absence and the presence of 25 mM-MgCl₂ are compared in Fig. 1; the cultures were stained with neutral red 7 days after infection, and photographed on day 9.

Table 1. Increase of plaque count of adenovirus 5 on HeLa cell monolayers overlaid with normal Eagle’s medium and Eagle’s medium supplemented with various concentrations of MgCl₂

<table>
<thead>
<tr>
<th>MgCl₂ concentration, mM</th>
<th>Day 0</th>
<th>12</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>42</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td>11</td>
<td>9</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>5</td>
<td>19</td>
<td>20</td>
<td>14</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>18</td>
<td>36</td>
<td>38</td>
<td>26</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>31</td>
<td>40</td>
<td>41</td>
<td>29</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>46</td>
<td>49</td>
<td>46</td>
<td>36</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>41</td>
<td>49</td>
<td>50</td>
<td>49</td>
<td>40</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>11</td>
<td>47</td>
<td>50</td>
<td>50</td>
<td>49</td>
<td>40</td>
<td>40</td>
<td>37</td>
</tr>
<tr>
<td>12</td>
<td>52</td>
<td>51</td>
<td>50</td>
<td>49</td>
<td>40</td>
<td>40</td>
<td>37</td>
</tr>
</tbody>
</table>

(Estimated 50 p.f.u. seeded/dish on day 0.) Values in the table are the mean numbers of plaques/dish for four dishes.

The plaques formed in the presence of MgCl₂ are clearer and at least twice as large as those formed in untreated cultures. Pre-treatment of monolayers with MgCl₂ for 12 to 24 hr before infection, either with or without post-treatment, had no additional effect on adenovirus plaque formation. MgCl₂ also greatly enhanced the rate of production and the size of adenovirus 5 plaques in HeLa cells grown at either 39 or 31°C.
A similar effect was shown by 25 mM-MgCl₂ on plaque production of adenovirus types 1, 2 and 12 on HeLa cells. The development of plaques by adenoviruses type 1 and 2 follows a similar course to that of adenovirus type 5, but adenovirus 12 plaques take longer to develop. In the presence of MgCl₂, the adenovirus 12 plaques first appeared around the 7th day after infection, and the maximum plaque count was attained at 12 to 14 days, while in the absence of MgCl₂, the plaques first appeared at 9 to 10 days postinfection, and the count continued to rise thereafter. In this case it was difficult to measure the maximum plaque count since the HeLa cells tend to degenerate after 15 days. The infectivities of adenovirus 12 stocks measured at 14 days in the presence of 25 mM-MgCl₂ were consistently 5 to 10 times higher than those measured in its absence. The plaques attained a diameter of 1 to 1.5 mm. in the presence of MgCl₂, approximately twice the size of those formed in its absence.

Besides MgCl₂, other salts, at a concentration of 25 mM were tested for their effect on plaque formation by adenovirus type 5. Plaque formation was enhanced by CaCl₂ but the cells tended to clump in treated cultures, and when stained with neutral red these cultures lysed rapidly. Plaque formation was also enhanced by MgSO₄ but the rate of formation was reduced compared with cultures containing MgCl₂. Neither NaCl nor KCl had any enhancing effect upon adenovirus plaque production.

The effect of high levels of MgCl₂ on the plaques formed by small plaque variants of polyoma and SV40 differed from that produced on adenoviruses. SV40 plaque formation on BSC-1 cells was not enhanced by 25 mM-MgCl₂, and in fact smaller plaques resulted, although counts were not reduced. The rate of polyoma virus plaque formation was markedly depressed by 25 mM-MgCl₂, and in addition, both the size of the plaques and the final plaque counts were reduced about twofold.

The enhancement of adenovirus plaque formation by MgCl₂ could result from an increase in the yield of virus at high Mg²⁺ concentration. In order to test this possibility, the infectivities were measured for intracellular and extracellular virus in cells infected with adenovirus at input multiplicities ranging from 0.05 to 5 p.f.u./cell. When cells were infected at low multiplicities with adenovirus type 5 or type 12 the levels of extracellular virus were 10- to 30-fold higher in the cultures which contained 25 mM-MgCl₂, when measured at 36, 48 and 60 hr after infection, while the levels of intracellular virus were not affected.

In order to determine whether the rate of virus release was enhanced by MgCl₂, or whether virus was released earlier in treated cells, one-step growth experiments were carried out with adenovirus type 5. Cells were infected at a multiplicity of 5 p.f.u./cell and virus was allowed to adsorb for 90 min. Infective centre assays indicated that 90 to 95 % of the cells were infected under these conditions. After adsorption, the monolayers were washed twice with Eagle's medium to remove excess virus, and a liquid overlay consisting of Eagle's medium containing 2 % foetal calf serum was added. To half of the cultures, 25 mM-MgCl₂ was added immediately. Thereafter, cells and extracellular medium were sampled at regular intervals up to 40 hr and assayed for infectivity. Cells were frozen and thawed three times to release virus, and the medium was spun at 2000 rev./min. for 10 min. to remove floating cells and debris. The one-step growth curves obtained in one experiment with adenovirus 5 are shown in Fig. 2. The intracellular growth of adenovirus 5 is similar in both low and high Mg²⁺, with the increase of infectivity beginning 16 hr after infection. The release of virus into the medium started at 26 hr, both with and without MgCl₂, but the rate of virus release was much greater in the medium containing 25 mM-MgCl₂. Pre-treatment for 20 hr with MgCl₂, in addition to post-treatment did not alter this effect.
A number of important points arise from this work. Plaque formation by adenoviruses on HeLa cells is completed much earlier and plaque size is increased when 25 mM-MgCl₂ is incorporated in the agar-overlay medium. As a result, the plaque assay of human adenoviruses on HeLa cells is made easier and more rapid. The improved plaque assay reported here is considerably more rapid than those reported by others using HeLa, KB and other human cells (Green, Pifia & Kimes, 1967; Kjellén, 1961; Ledinko, 1967; Rouse, Bonifas & Schlesinger, 1963) and just as rapid as that reported by Russell et al. (1967). However, preliminary observations indicate that MgCl₂ may be less effective in other cell types. Adenovirus 5 plaque formation on human embryo lung cells was enhanced, but the cells lysed within 7 days in MgCl₂. Plaque production by adenovirus 5 in KB cells was not enhanced by 25 mM-MgCl₂, and the cells degenerated in 7 days.

![Graph](image)

Fig. 2. Effect of 25 mM-MgCl₂ on the cell-associated and extracellular yields of adenovirus type 5 in HeLa cells. O—O, cell-associated virus, no MgCl₂; •—•, cell-associated virus, 25 mM-MgCl₂; Δ—Δ, extracellular virus, no MgCl₂; ▲—▲, extracellular virus, 25 mM-MgCl₂.

In liquid-overlaid cultures of infected HeLa cells, more virus was released into the medium in the presence of 25 mM-MgCl₂, while there was little or no increase in the intracellular level of virus. During a single growth cycle of adenovirus 5, the rate of virus release was increased greatly by 25 mM-MgCl₂. These findings support the view that enhancement of plaque formation and size is due to more extensive spread of virus from cell to cell as a result of the more rapid release of virus in the presence of 25 mM-MgCl₂. The results agree with the explanation put forward for the enhancing effect of MgCl₂ on poliovirus (Wallis & Melnick, 1962) and rhinoviruses (Fiala & Kenny, 1967). Preliminary results indicate that in addition to the effect on virus release, MgCl₂ also promotes attachment and penetration of adenovirus type 5 to HeLa cells. However, the effect is small, and since uptake of adenovirus 5 into HeLa cells is very rapid without MgCl₂, it is unlikely that the effect contributes to plaque enhancement.

The reason for the adverse effect of MgCl₂ on SV40 and polyoma virus plaque formation is not known. It is unlikely to be due simply to a cytotoxic effect, since the cells remained...
viable even in 40 mM-MgCl₂, as judged by their ability to retain neutral red. The effect could be due either to a decreased rate of virus release, to inactivation of extracellular virus by the high concentration of MgCl₂, or to increased aggregation of the released virus by MgCl₂.

The mechanism by which MgCl₂ promotes adenovirus release is under study. Whatever the mechanism, the enhancement of adenovirus plaque formation in HeLa cells by MgCl₂ is of great practical importance, since it is now much easier to investigate the effects of various agents on the viability and other properties of human adenoviruses.

I thank Professor J. H. Subak-Sharpe and Dr Meera Gharpure for valuable discussion during the preparation of this paper, and Mrs Sylvia McDonald for excellent technical assistance.

M.R.C. Virology Unit
Institute of Virology
Church Street
Glasgow

REFERENCES


(Received 29 June 1970)