Clonal Variation of the Mouse Cells in the Endogenous C-type
Virus Induction by 5-iodo-2'-deoxyuridine

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SUMMARY

Using an MLg cell line originating from a single newborn ddY mouse, N-tropic C-type virus able to infect the cells of origin was induced by 5-iodo-2'-deoxyuridine (IUDR). All the clones isolated from the MLg cell line released the virus after IUDR-treatment. There was more than a 100-fold difference in the virus inducibility between the highly and lowly inducible clones. The [125I]-IUDR incorporation was, however, the same for the both types of clones.

When the induced virus was titrated in the S+L− C182 cells, the XC plaque titre was about tenfold higher than murine sarcoma virus rescue focus titre.

INTRODUCTION

MLg cells originating from a single newborn ddY mouse came to release C-type virus ‘spontaneously’ after a long cultivation in vitro (Yoshikura & Hirokawa, 1974). Such virus-producing cells consisted of three types. Namely, when the cells were cloned and tested for the presence of infectious centres on to S+L− C182 cells (Bassin, Tuttle & Fischinger, 1970, some clones produced rescue foci of the transformed cells (due to activation of mouse sarcoma virus) and XC plaques (‘F+P+’), others produced XC plaques but not rescue foci (‘F−P+’), and still others produced neither XC plaques nor rescue foci but were positive for the polykaryon formation when the cells were co-cultivated with the XC cells (‘F−P−’) (Yoshikura & Hirokawa, 1974). The formation of rescue foci may be considered as a test of the virus function which is necessary for the rescue of murine sarcoma virus (MuSV) from the S+L− cells, and the XC plaque formation as a test of the production of infectious virus.

These previous data suggested that the cells originating from the same germinal cell (as the cells originate from a single mouse) were heterogeneous with respect to the ‘spontaneous’ C-type virus activation. The data presented here show that clonal variation also exists in the case of C-type virus induction by 5-iodo-2'-deoxyuridine (Lowy et al. 1971), but in a different way from the ‘spontaneous’ induction.

METHODS

Cells. The MLg cell line originating from the lung tissue of a single newborn ddY mouse was used; the cell line was N-type according to the classification by Hartley, Rowe & Huebner (1970) as described by Yoshikura & Hirokawa (1974). The origins of the XC, the S+L− C182, and the B-type C57BL/6 mouse YH-7 cell lines were already described (Yoshikura & Hirokawa, 1974).
The culture medium consisted of 9 parts autoclavable modified Eagle's MEM (Nissui Co.) and 1 part calf serum inactivated by heating at 56 °C for 30 min.

Cloning. The cells were plated in 60 mm plastic Petri dishes, 250 to 500 cells per dish. After 2 weeks visible colonies appeared. Culture medium was discarded, and soft agar medium (0.6%) overlaid on to the cells. The colonies were isolated under the microscope with a Pasteur pipette through the agar layer, and further cultivated in 35 mm plastic Petri dishes.

Infectious centre assay by the focus rescue and plaque assays in S+L− cells. The cells were trypsinized, and a known number of cells (10^8 to 10^4 per dish) were plated on to S+L− cells cultivated in 60 mm Petri dishes. The rescue MSV foci (equivalent to cytopathic plaques; Bassin et al. 1971) were counted on the fifth day, and the cultures were further submitted to the XC test (Rowe, Pugh & Hartley, 1970). The value obtained by dividing the number of XC plaques or rescue foci by the number of the cells inoculated will be called XC plaquing or rescue focus forming efficiency (abbreviated to P/N or F/N, respectively), and the value obtained by dividing the number of rescue foci by the number of the XC plaques will be called rescue focus-XC plaques ratio (abbreviated to F/P).

Titration of induced virus. Following IUdR treatment (see results) the medium of the treated cells was tested for free virus, firstly, in the mouse cells by the XC test (Rowe et al. 1970) and, secondly, in S+L− cells by counting of the rescue foci (Bassin et al. 1971) which were performed as described previously (Yoshikura, 1973). The infected S+L− cells were further submitted to the XC test after the rescue foci were counted.

RESULTS

C-type virus induction in cloned MLg cells

From normal MLg cells at the 45th passage, 18 clones were obtained, and used for the virus activation by 5-iodo-2'-deoxyuridine (IUdR) about 2 weeks after the isolation. The cells were inoculated into 60 mm dishes in an amount of 10^5 cells/dish. The next day, the medium was replaced by the medium containing IUdR at a concentration of 25 μg/ml.
**Endogenous mouse C-type virus**

Table 1. **Induction of C-type virus from highly inducible and lowly inducible clones and $[^{125}I]$-IUdR incorporation in these cells**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number of plaques/dish*</th>
<th>$[^{125}I]$-IUdR incorporation† (ct/min/10⁵ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI-1</td>
<td>Expt. 1 (27‡) 1, 1, 0</td>
<td>4, 1, —§ 33, 45, 49 36.9 x 10⁴</td>
</tr>
<tr>
<td>CI-3</td>
<td>Expt. 2 (29) 2, 0, 1</td>
<td>1, 0, — 15, 20, — 32.0 x 10⁴</td>
</tr>
<tr>
<td>CI-9</td>
<td>Expt. 3 (49) 36, —, —</td>
<td>302, —, — 264, 282, — 30.8 x 10⁴</td>
</tr>
<tr>
<td>CI-16</td>
<td>—, 104, —</td>
<td>280, 267, — 150, 192, 207 —</td>
</tr>
</tbody>
</table>

* The cells (10⁵ cells/60 mm Petri dish) were treated with 25 μg/ml of IUdR for 24 h. The IUdR was removed and untreated, uncloned MLg cells (10⁵ cells/dish) were overlaid on to the cells. Three days later, the cultures were divided into three. After incubation for 3 further days, the cultures were submitted to the XC test.

† The cells (10⁵ cells/60 mm Petri dish) were cultured in the medium containing $[^{125}I]$-IUdR (0.6 μg/ml, 5.2 μCi/ml) for 24 h. The radioactivity incorporated was measured by a crystal well-type gamma counter, after acid soluble fraction being extracted. This experiment was performed in parallel with Expt. 2.

‡ Days after isolation of the clones. § Cultures lost.

Twenty-four hours later, the medium was replaced by IUdR-free medium. Untreated uncloned MLg cells at a concentration of 10⁵ cells/dish were overlaid on to the IUdR-treated cells. Three days later, the cultures were split into three. After incubation for a further 3 days, two of the cultures were submitted to the XC test. The XC plaque number (the mean of the two values) thus obtained (I) was considered as a measure of the inducibility of the endogenous virus. One remaining culture was cultivated for a further two passages, and tested for infectious centres in S + L − C182 cells by the two tests described, namely the formation of the rescue foci and as XC plaques (at this time, all the cultures developed polygonkaryons confluently in the XC test). The XC plaquing efficiency (P/N) and the rescue focus-XC plaque ratio (F/P) were determined (see Methods). In Fig. 1, the F/P ratio was plotted against inducibility (I). There was more than 100-fold difference in the inducibility between highest and lowest inducible clones, while the F/P ratio was in the relatively narrow range of 0.06 to 0.3. The P/N was in the range of 0.12 to 0.24. That is, in spite of the large variation in the inducibility, all the clones were fairly alike with respect to the rescue focus and XC plaque formations in the infectious centre assay in the S + L − cells.

Highly inducible clones, CI-9 and CI-16 and lowly inducible clones, CI-1 and CI-3, were tested twice further for inducibility. Table 1 (first 2 columns) shows that the different inducibility in these clones was reproducible. After further passages, however, the lowly inducible clones tended to be more inducible (cf. third column in Table 1).

The $[^{125}I]$-IUdR incorporation was measured in these clones. The cells were inoculated at a concentration of 10⁵ cells/60 mm Petri dish. The next day, the culture medium was replaced with the medium containing $[^{125}I]$ (The Radiochemical Centre Ltd, Amersham, 0.6 μg/ml, 5.2 μCi/ml). After 24 h contact, the cells were trypsinized, treated with 5% trichloroacetic acid at 4 °C for 45 min, washed once with ice-cold phosphate buffered saline (PBS), and suspended in 1 ml of PBS. The radioactivity was measured by a crystal well-type gamma counter. The radioactivity incorporated/cell was similar in the highly inducible CI-9 and the lowly inducible CI-1 and CI-3 (Table 1, the last column).
Fig. 2. Infectious centre assay in the S + L− cells of the spontaneously virus producing MLg cell clones and the subclones of V-Cl-12, an I UdR-induced MLg cell clone. ▲, clones of spontaneously virus-producing MLg cells at 76th passage; ○, subclones of V-Cl-12. Clones producing detectably no foci or plaques (< 0.001) are 'F-P-'. The straight lines indicate the focus-plaque ratio, F/P, to give 1.0, 0.1, 0.09 and 0.01.

**Infectious centre assay of the subclones derived from an I UdR-induced MLg clone**

A virus-producing clone, V-Cl-12, was obtained by treating MLg clone Cl-12 (cf. Fig. 1) with 25 μg/ml of I UdR for 24 h (the cells were not overlaid with untreated MLg cells after I UdR in this experiment). From this clone, 15 subclones were isolated. Each of them was tested for the infectious centre as rescue foci and XC plaques in the S + L− cells. The number of cells inoculated was 10⁸ to 10⁹ cells/dish. All the clones produced both rescue foci and XC plaques. The XC plaquing efficiency was around 0.1 for all the clones, while the rescue focus XC plaque ratio was distributed in the range of 0.001 to 0.1 (Fig. 2). The wide variation of the F/P ratio was probably due to the heterogeneous population of the induced virus (see below).

In order to compare I UdR induction and 'spontaneous' induction, MLg cells at the 76th passage which had come to release the virus 'spontaneously' were cloned and assayed for infectious centres in S + L− cells. Four clones were positive for rescue foci as well as XC plaques ('F + P+'); five were negative for rescue foci but positive for XC plaques ('F−P−'); six were negative for both rescue foci and XC plaques ('F−P−') (Fig. 2). The latter observation confirms the author's previous data (Yoshikura & Hirokawa, 1974).

**Titration of the induced virus**

V-Cl-1, V-Cl-12 and V-MLg were obtained by treating the lowly inducible clone Cl-1, the highly inducible clone Cl-12 and uncloned MLg cells, respectively, with I UdR (25 μg/ml). The culture fluids of these cells were titrated in the S + L− cells both as rescue foci and XC plaques. Table 2 shows that the XC plaque titre was higher than the rescue focus titre.
**Table 2. Titration of the culture fluid of the I UdR-induced virus producing cells***

<table>
<thead>
<tr>
<th>Virus source</th>
<th>Dilution</th>
<th>Rescue foci</th>
<th>XC plaques</th>
<th>MLg (N-type)</th>
<th>YH-7 (B-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-MLg</td>
<td>1/2</td>
<td>19, 13†</td>
<td>58, 72</td>
<td>171, 131</td>
<td>6, 4</td>
</tr>
<tr>
<td></td>
<td>1/10</td>
<td>4, 2</td>
<td>15, 25</td>
<td>62, 33</td>
<td>NC‡</td>
</tr>
<tr>
<td></td>
<td>1/50</td>
<td>0, 0</td>
<td>4, 7</td>
<td>8, 6</td>
<td>NC</td>
</tr>
<tr>
<td>V-Cl-1</td>
<td>1</td>
<td>10, 7</td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>3, 0</td>
<td>37, 48</td>
<td>ND</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>0, 0</td>
<td>15, 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-Cl-12</td>
<td>1</td>
<td>3, 1</td>
<td>68, --</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>0, 0</td>
<td>16, 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The culture fluid which had been in contact with the cells for 24 h was harvested and stock frozen at -70 °C until titration. In the S+L- C182 cells, the cultures were submitted to the XC test after the rescue foci were counted.

† Number of rescue foci or XC plaques per dish. †† Not counted. § Not done. || Cultures lost.

by about tenfold for all these three samples. It should be noted that in the same titration, Friend leukaemia virus produced the same number of rescue foci as XC plaques, and it was suggested that such a disparity in the rescue focus titre and XC plaque titre of the induced virus was due to the presence of two different types of viruses: one is efficient and the other inefficient in the rescue focus formation, and the latter is in excess over the former (Yoshikura & Hirokawa, 1974).

The induced virus was N-tropic, since the infectivity in N-type MLg cells was about 30-fold higher than in B-type YH-7 cells.

**DISCUSSION**

There was a large variation in the inducibility of the C-type virus among the MLg clones. The difference between clones probably originates in the processes which occur after I UdR incorporation into DNA, since the incorporation of [125I]-I UdR was almost the same in the highly and lowly inducible clones. However, the nature of the variation remains obscure. It is also unknown why the lowly inducible clones tended to be more inducible after further passages. Repeated cloning may be necessary for obtaining more stable clones.

In spite of this difference in the inducibility, all the clones, after I UdR induction, showed a similar value of XC plaquing efficiency and rescue focus-XC plaque ratio in the infectious centre assay in the S+L- cells. This suggests that each MLg cell inherits the same endogenous virus genomes at least with respect to the rescue focus and XC plaque-forming capacities of the endogenous virus. This view is also supported by the fact that even the 'F—P—' clones of 'spontaneously' induced MLg cells (Yoshikura & Hirokawa, 1974) produced rescue focus as well as XC plaque-forming virus although in an extremely small amount (H. Yoshikura, unpublished data). This is in accordance with the conclusion derived from the study of AKR mouse cells (Rowe et al. 1971) that probably all the cells in the mouse cell lines carry the full viral genome in an unexpressed form.

The subclones of I UdR-induced clone, V-Cl-12, all produced both rescue foci and XC plaques in the infectious centre assay in the S+L- cells, although there was a wide variation in the rescue focus-XC plaque ratio, the XC plaquing efficiency being nearly the same.
for all the clones. On the other hand, from the ‘spontaneously’ virus-inducing MLg cells, three types of clones, ‘F+P+’, ‘F−P+’ and ‘F−P−’ clones were isolated. Thus, the spontaneous virus activation and IUdR-induced virus activation were not superimposable. The phenomenon may be interpreted as follows. In the ‘spontaneous’ virus activation, ‘F−P−’ or ‘F−P+’ clones appear first predominantly and the ‘F+P+’ type will appear late. In the IUdR virus activation, most virus-producing cells were not activated but infected with the virus activated in ‘F+P+’ cells. Thus, the majority of the cells were those infected with the virus whose infectivity and replication rate was high and the ‘F−P−’ cells, even if they existed at the beginning, were diluted out after several passages and could not be isolated as clones.

Finally, the induced C-type virus, induced from AKR mouse cells (Lowy et al. 1971), did replicate in the homologous cells. If the expression of the endogenous virus genomes is suppressed by some repressor substance, as proposed by Todaro & Huebner (1972), the induced virus could not have replicated in the homologous cells. That is, after the integration of its genomes with cellular DNA, its transcription would have been suppressed by the repressor. This may indicate that in the case of mouse tropic virus, but not in that of exotropic virus (Aaronson & Dunn, 1974), the expression of the endogenous virus genomes is not suppressed by much repressor substance, or, if the repressor exists, that some mechanism exists which prevents the repressor from acting on the provirus of the induced virus genomes.

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REFERENCES


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