Plaque Formation by a Host Range Mutant of Vaccinia Virus in Non-permissive Cells Co-infected with Yaba Virus

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SUMMARY

A host-dependent conditional lethal mutant of vaccinia virus strain DI was rescued to form plaques in a non-permissive cell line JINET co-infected with Yaba virus, a poxvirus serologically distant from the rescued virus. The efficiency of plaque formation under optimal conditions in this system was comparable to that in Vero cells which were permissive for the mutant. The plaque number of the mutant in JINET cells was influenced greatly by the multiplicity of Yaba virus, the interval between inoculations with DI and Yaba virus and the maintenance medium for pre-infection of cells with Yaba virus. The plaque formation by DI in JINET cells was suppressed or inhibited when the duration of pre-infection with high multiplicities of Yaba virus was prolonged. Thus, Yaba virus possessed both rescuing and inhibitory activities on DI and the plaque number of DI in doubly infected cells was thought to be determined by the balance between the two activities. Ultraviolet light-inactivated Yaba virus retained rescuing activity on DI in JINET cells.

INTRODUCTION

Sambrook et al. (1966) and Fenner & Sambrook (1966) isolated and characterized a group of host-dependent conditional lethal mutants of rabbitpox virus which had interesting characteristics. These mutants showed a wide variety of phenotypes in a non-permissive host cell line PK-2a. Nevertheless they did not complement each other in these cells and all of the 34 mutants they isolated belonged to a single complementation group (Fenner & Sambrook, 1966). This is in contrast to the results obtained with 18 temperature-sensitive mutants of the same virus which complemented each other in most crosses, although they showed little phenotypic diversity (Padgett & Tomkins, 1968). One of the host-dependent conditional lethal mutants, u6/2, was shown to be defective at different stages of its multiplication in three different non-permissive host cell types (Sambrook et al. 1965). The peculiarities of this group of host-dependent conditional lethal mutants of rabbitpox virus suggested that they were defective in some control function (Sambrook et al. 1965; Fenner & Sambrook, 1966). The defective function of u6/2 was complemented in PK-2a cells by a non-restricted strain of rabbitpox virus (McClain & Greenland, 1965).

In a previous paper (Tsuchiya & Tagaya, 1977), it was shown that the DI strain of a host-dependent conditional lethal mutant of vaccinia virus (Tagaya et al. 1961) and several strains of the rabbitpox mutants of Fenner & Sambrook (1966) were rescued in a non-permissive host cell line JINET by Yaba virus, a poxvirus serologically distant from ortho-
poxviruses. It was also shown that DIs did not complement any one of the rabbitpox mutants tested and that DIs and the rabbitpox mutants had the same host range as far as our tests showed. These facts suggested that DIs also belonged to the same complementation group as that of the rabbitpox mutants. Studies on the defective function and the rescue phenomena observed with these orthopoxvirus mutants may contribute towards the understanding of the mechanisms (Sambrook et al. 1965; Fenner & Sambrook, 1966) controlling the replication of orthopoxviruses at various stages. Information on this subject is rather scanty and clarification is needed.

This communication described the rescue of DIs to form plaques efficiently in a non-permissive host cell line JINET co-infected with Yaba virus. The rescue phenomenon was characterized by analysing the conditions for plaque formation by DIs in JINET cells co-infected with Yaba virus.

**METHODS**

**Cell cultures.** A cynomolgus monkey kidney cell line JINET and an African green monkey kidney cell line Vero were grown in Eagle's minimal essential medium (MEM) containing 5% bovine serum and 0.075% (JINET) or 0.1% (Vero) sodium bicarbonate. Both were maintained in MEM containing 2% bovine serum and 0.15% sodium bicarbonate unless otherwise stated.

**Viruses.** The DIs strain of a host-dependent conditional lethal mutant of vaccinia virus was isolated from its parent strain Dairen I after repeated passages of the latter virus through 1-day-old eggs (Tagaya et al. 1961). DIs grows in chorioallantoic membranes (CAM) of embryonated eggs, primary chick embryo fibroblasts and primary and continuous lines of African green monkey kidney cells but not in primary or continuous lines of cynomolgus monkey kidney cells, human embryo lung cells, a pig kidney cell line PK-15 or a rabbit kidney cell line RK-13 (Tagaya et al. 1961; Tagaya et al. 1972; Tsuchiya & Tagaya, 1977). All the cell types listed above support the growth of Dairen I. DIs was propagated in CAM and assayed in Vero cells by the plaque method. Host-dependent conditional lethal mutants of rabbitpox virus u6/2, u22, u23 and p123 were also used in part of the present study. These were generous gifts from Dr F. Fenner, Canberra. The properties of these mutants have been described (Fenner & Sambrook, 1966). They were propagated and assayed in Vero cells. Dairen I was grown in CAM and assayed in JINET cells by the plaque method. Yaba virus was propagated and assayed in JINET cells as described previously (Tsuchiya et al. 1969). Yaba virus was purified by sucrose density gradient centrifugation before use in u.v.-inactivation experiments. Yaba virus causes a mild infection in JINET cells but does not produce plaques in JINET or Vero cells under our experimental conditions. Yaba virus-infected cells can be maintained in culture for 2 weeks.

**Plaque assay of the mutants.** Virus appropriately diluted in maintenance medium was inoculated in 0.2 ml amounts into drained 2 oz prescription bottle cultures of Vero cells and allowed to adsorb for 2 h at room temperature. Agar overlay (5 ml) was then added to the cultures which were then incubated at 36 °C. Agar overlay medium consisted of medium 199 containing 1% Noble agar (Difco), 2% bovine serum, 0.22% sodium bicarbonate and 800 μg/ml DEAE-dextran (Pharmacia). Plaques were counted after staining monolayers with neutral red 5 to 7 days after inoculation. When the mutants were assayed in JINET cells co-infected with Yaba virus, the concentration of DEAE-dextran in agar overlay medium was 500 μg/ml and plaques were counted 5 days after inoculation with the mutants.
Table 1. Plaque assay of host-dependent conditional lethal mutants of vaccinia and rabbitpox viruses in Vero and normal and Yaba virus-co-infected JINET cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Normal JINET</th>
<th>Co-infected JINET (a)</th>
<th>Vero (b)</th>
<th>a/b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia</td>
<td>DI5</td>
<td>&lt;5 × 10⁴</td>
<td>9·5 × 10⁷</td>
<td>9·3 × 10⁷</td>
<td>1·02</td>
</tr>
<tr>
<td>Rabbitpox</td>
<td>u6/2</td>
<td>&lt;5 × 10⁴</td>
<td>6·7 × 10⁷</td>
<td>1·7 × 10⁷</td>
<td>0·39</td>
</tr>
<tr>
<td></td>
<td>u22</td>
<td>&lt;5 × 10⁴</td>
<td>7·2 × 10⁶</td>
<td>2·9 × 10⁶</td>
<td>0·25</td>
</tr>
<tr>
<td></td>
<td>u23</td>
<td>&lt;5 × 10⁴</td>
<td>5·5 × 10⁶</td>
<td>1·8 × 10⁶</td>
<td>0·31</td>
</tr>
<tr>
<td></td>
<td>p123</td>
<td>&lt;5 × 10⁴</td>
<td>5·8 × 10⁶</td>
<td>1·5 × 10⁶</td>
<td>0·39</td>
</tr>
</tbody>
</table>

RESULTS

Comparative plaque assay of DI5 and rabbitpox mutants in JINET cells co-infected with Yaba virus and in Vero cells

DI5s was assayed in JINET cells co-infected with Yaba virus and in Vero cells and the titres were compared in both assay systems. The rabbitpox mutants were also assayed in both systems to determine whether the plaque rescue was a general phenomenon with Yaba virus and the members of the complementation group of Fenner & Sambrook (1966) to which DI5 also belong. The multiplicity of Yaba virus was 0·4 focus-forming units (f.f.u.)/cell and the mutants and Yaba virus were inoculated simultaneously into JINET cells. The mutants were also inoculated into JINET cells in the absence of Yaba virus and into Vero cells. Plaques appeared 3 to 4 days after inoculation in both Vero and co-infected JINET cells. Five days after inoculation the plaque diam. was about 1 mm. As shown in Table 1, the plaque titre of DI5 in co-infected JINET cells was comparable to that in Vero cells. The titres of the rabbitpox mutants in co-infected cultures were 1/4 to 1/5 of those in Vero cells. DI5 and the rabbitpox mutants did not produce plaques in normal JINET cells nor did Yaba virus alone under our experimental conditions. After neutralization by a cowpox virus antiserum, DI5 failed to produce plaques in co-infected cells (data not shown).

Inhibition of plaque formation and growth of Dairen I
in JINET cells pre-infected with Yaba virus

In a previous paper (Tsuchiya & Tagaya, 1970), we reported that plaque formation by some super-infecting viruses including vaccinia virus was completely inhibited in JINET cells pre-infected with Yaba virus. Our present finding that orthopoxvirus mutants were rescued to form plaques in JINET cells co-infected with Yaba virus seemed to be incompatible with the previous result. It was decided therefore to re-examine the inhibitory effect of Yaba virus pre-infection on plaque formation by Dairen I. JINET cells were infected with Yaba virus at various multiplicities and incubated at 36 °C for 3 days. Appropriate dilutions of Dairen I were then inoculated into the pre-infected and normal JINET cells and the cultures were overlaid with agar medium after adsorption for 2 h at room temperature. Plaques were counted 3 days after inoculation with Dairen I. As shown in Fig. 1, Yaba virus at a multiplicity of 0·4 f.f.u./cell completely inhibited plaque formation by Dairen I. The degree of inhibition was proportional to the multiplicity of Yaba virus and the multiplicities of 0·013 or smaller had no effect on the plaque number of Dairen I. The inhibition of plaque formation also paralleled the inhibition of the growth of Dairen I (Fig. 1). Thus the results reported in the previous paper are confirmed and it was also shown
that the inhibition of plaque formation was based on the inhibition of vaccinia virus replication.

Our preliminary experiments showed that the plaque number of DIIs in mixedly infected JINET cells varied greatly under various experimental conditions and sometimes plaques did not appear at all. It was anticipated that under certain conditions plaque formation by DIIs in JINET cells infected with Yaba virus might be inhibited like that of Dairen I virus. Our next concern was to clarify the factors influencing plaque formation by DIIs in co-infected JINET cells.

Effect of multiplicity of Yaba virus and period of pre-infection on the plaque number of DIIs in JINET cells

Yaba virus was serially diluted in two-fold steps and inoculated into 2 oz prescription bottle cultures of JINET cells 1, 2 and 3 days before inoculation with DIIs. After incubation at 36 °C, DIIs was assayed in these pre-infected cultures. In a parallel series of cultures, Yaba virus and DIIs were inoculated simultaneously. The results depicted in Fig. 2(a) and (b) show that the multiplicity and the duration of pre-infection with Yaba virus influenced...
A vaccinia virus mutant and Yaba virus

Fig. 2. Effect of multiplicity and duration of pre-infection with Yaba virus on plaque formation by DIs in JINET cells. (a) The plaque number of DIs obtained in cultures inoculated with Yaba virus 3 (□—□), 2 (■—■), 1 (○—○) and 0 (●—●) days before inoculation with DIs is plotted against multiplicity of Yaba virus. (b) The plaque number of DIs obtained in the same experiments as (a) is plotted against the duration of pre-infection with Yaba virus.

the plaque number of DIs. Plaque formation by DIs was completely inhibited in cultures infected with Yaba virus at a multiplicity of 0.4 f.f.u./cell for 2 to 3 days. As shown in Fig. 2(b), the plaque number decreased according to the increase in pre-infection time when cultures were inoculated with Yaba virus at multiplicities of 0.05 to 0.4 f.f.u./cell. On the other hand, plaque number increased as the pre-infection period increased with 0.025 f.f.u./cell or smaller multiplicities. The plaque numbers were maximal at multiplicities of 0.2, 0.1, 0.05, and 0.025 f.f.u./cell of Yaba virus when pre-infection periods were 0, 1, 2 and 3 days, respectively. The results of these experiments showed that Yaba virus had both rescuing and inhibitory effects on DIs.

**Effect of Yaba virus on plaque formation by DIs and Dairen I in Vero cells**

The effect of pre-infection with Yaba virus on plaque formation by DIs and Dairen I was also examined in Vero cells which were permissive for DIs but rather insensitive to Yaba virus. Yaba virus at various multiplicities was inoculated into Vero cell cultures and the infected cultures were incubated at 36 °C for 2 days. Then, DIs and Dairen I were assayed in the pre-infected cultures and in uninfected control cultures. As shown in Fig. 3, Yaba virus at high multiplicities enhanced the plaque formation by DIs by up to threefold. The effect of Yaba virus on Dairen I was not as pronounced as with DIs. It should be noted that at no m.o.i. did Yaba virus inhibit plaque formation by either of the super-infecting
viruses. The plaque number of Dairen I was slightly enhanced at a multiplicity of Yaba virus of 0.05 f.f.u./cell. This enhancement was observed more than once. The plaque diam. of Dairen I was slightly but definitely larger in cultures inoculated with the highest multiplicity (0.4 f.f.u./cell) of Yaba virus than in control cells.

**Influence of maintenance media used during pre-infection**

The results described in the preceding section indicated that Yaba virus had both a rescuing and an inhibitory effect on DIs and an attempt was made to separate the two activities. The data thus far obtained suggested that the conditions which favoured the replication of Yaba virus in co-infected cells were suppressive for plaque formation by DIs. Yaba virus replicates poorly in cells maintained in Earle's salt solution with 0.5% lactalbumin hydrolysate (LE medium; Y. Tsuchiya & I. Tagaya, unpublished results). The plaque number of DIs in cultures maintained in LD medium during pre-infection was, therefore, compared with that in cultures maintained in MEM. JINET cell cultures inoculated with Yaba virus at appropriate multiplicities were divided into two groups and the one was maintained in LE medium and the other in MEM. After incubation at 36 °C for 3 days, the cultures were inoculated with appropriately diluted DIs and the plaques were developed. As indicated in Table 2, the plaque number in cultures inoculated with 0.1
Table 2. Plaque production by DIIs in Yaba virus-infected JINET cells maintained in different media during pre-infection*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dose of DIIs† (p.f.u./bottle)</th>
<th>M.o.i. of Yaba virus (f.f.u./cell)</th>
<th>LE medium</th>
<th>MEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200</td>
<td>0.1</td>
<td>68.5</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.025</td>
<td>87.0</td>
<td>93.5</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>0.1</td>
<td>22.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.025</td>
<td>29.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

* Pre-infection was for 3 days at 36 °C.
† Assayed in Vero cells.

Fig. 4. Effect of u.v.-irradiation on infectivity and rescuing ability of Yaba virus. Yaba virus infectivity (●—●). Rescuing ability in terms of plaque number of DIIs (○—○).

F.f.u./cell of Yaba virus and maintained in LE medium was 20 to 30 times greater than that in cultures maintained in MEM. On the other hand DIIs produced plaques at almost the same efficiency in pre-infected cells maintained in either MEM or LE medium when the multiplicity of Yaba virus was 0.025 f.f.u./cell.

**Distinction between rescuing activity and infectivity of Yaba virus by u.v.-irradiation**

Purified Yaba virus was diluted in PBS so that inoculation of unirradiated virus suspension into 2 oz prescription bottle cultures of JINET cells resulted in a m.o.i. of 0.4 f.f.u./cell. The virus suspension was irradiated by a u.v. lamp (10W) from a distance of 20 cm for
Days after inoculation with DIs

Surviving infectivity (log p.f.u./culture or 0.2 ml)

Fig. 5. Inactivation kinetics of DIs in JINET cells in maintenance medium at 36 °C. Inactivation kinetics of DIs in the maintenance medium (●—●). Interactivation kinetics of DIs in living JINET cells detectable by superinfection with Yaba virus (○—○). Inactivation kinetics of DIs detectable after disruption of infected cells by sonication (■—■).

various periods. Samples (0.2 ml) of unirradiated and irradiated Yaba virus together with appropriately diluted DIs were inoculated into JINET cell cultures. After adsorption for 2 h at room temperature, the cultures were overlaid with agar medium and incubated at 36 °C. The infectivity of unirradiated and irradiated Yaba virus was also assayed in JINET cells. As shown in Fig. 4, u.v.-irradiation up to 60 s did not significantly reduce the rescuing activity of Yaba virus while the infectivity of the virus was reduced by more than 5 logs in the same period.

Persistence of DIs in a rescuable form in JINET cells

DIs was diluted in 10-fold steps from $10^{-2}$ to $10^{-8}$ and 0.2 ml amounts of each dilution were inoculated into 2 oz prescription bottle cultures of JINET cells. After adsorption for 2 h at room temperature, the cultures were fed with maintenance medium and incubated at 36 °C. DIs in maintenance medium at $10^{-2}$ dilution was also dispensed in small test tubes and incubated at 36 °C. Some of the cultures inoculated with the various dilutions of DIs were withdrawn at intervals, super-infected with Yaba virus at a multiplicity of 0.4 f.f.u./cell and after 2 h overlaid with agar medium. At the same time intervals DIs suspended in maintenance medium at $10^{-2}$ and cultures inoculated with the same dilution of DIs were harvested and assayed for infectivity in JINET cells simultaneously infected with 0.4
A vaccinia virus mutant and Yaba virus

f.f.u./cell of Yaba virus. Dis-infected cells were disrupted by sonication before titration. Fig. 5 shows that an appreciable amount of Dis was rescued to form plaques in JINET cells when the cells were superinfected with Yaba virus up to 5 days after inoculation with Dis. The percentage survival of rescuable Dis in these cells which could be measured by superinfection with Yaba virus, 1, 3 and 5 days after inoculation with Dis were 23.0, 5.2 and 1.6, respectively. Fig. 5 also shows that Dis was inactivated more rapidly in JINET cells than in the maintenance medium at 36 °C. When Dis-infected cultures were sonicated and assayed for infectivity, the titre was about 1/1000 of that of rescuable Dis in living JINET cells. This suggests that Dis persisted in JINET cells in an eclipsed form.

DISCUSSION

The present communication shows that host-dependent conditional lethal mutants of orthopoxviruses were rescued by Yaba virus to form plaques in JINET cells which were non-permissive for the rescued viruses. In the previous reports (McClain & Greenland, 1965; Oda, 1965), the rescue phenomenon observed with poxviruses was demonstrable only by harvesting doubly infected cultures and distinguishing the viruses produced by passage in permissive cells. The system described in the present report enabled us to demonstrate the rescue phenomenon directly in mixedly infected cultures and also to quantify the rescued viruses in these cells. The efficiency of plaque formation by Dis in co-infected JINET cells under optimal conditions was comparable to that in Vero cells, suggesting that almost all the virions which could replicate in Vero cells were rescued in co-infected JINET cells. Thus, the combination of Dis in Yaba virus in JINET cells seems to be unique in that the rescue phenomenon may be detected at the level of the individual virions of the rescued virus.

The idea that the plaques were produced in mixed infections of JINET cells by Dis but not by Yaba virus is supported by the following facts: (1) Dis grows well in JINET cells co-infected with Yaba virus, the yield of the mutant in the co-infected cells being comparable to that in Vero cells (Tsuchiya & Tagaya, 1977). (2) The plaque-forming ability of Dis was neutralized by a cowpox virus antiserum as described in the present report and also inactivated by heating and u.v.-irradiation (Tsuchiya & Tagaya, 1977). Furthermore, u.v.-inactivation of Yaba virus infectivity by more than 5 logs did not cause a significant reduction in the plaque number in co-infected cultures (Fig. 4). (3) Yaba virus did not produce plaques in JINET cells when inoculated alone. It seems unlikely that the plaques were produced by recombinants between Yaba virus and Dis which could replicate in JINET cells because the product from the cultures with mixed infections was able to grow in JINET cells only in the presence of Yaba virus (Tsuchiya & Tagaya, 1977).

The plaque number of Dis in JINET cells was greatly influenced by the multiplicity of Yaba virus (Fig. 2), the interval between the inoculations with Dis and Yaba virus (Fig. 2) and the medium for maintenance of pre-infected cultures (Table 2). From the data presented in Fig. 2, Yaba virus was shown to have both rescuing and inhibitory activities on Dis in JINET cells. The plaque number of Dis in cultures with mixed infections seems to be determined by this rescue-inhibition balance. We think that the failure of Dis to produce plaques in JINET cells pre-infected with high multiplicities of Yaba virus for long periods is due to partially distinguished from the inhibitory one by using a maintenance medium deficient for the replication of Yaba virus during pre-infection (Table 2). The inhibitory activity of Yaba virus on plaque formation by Dis and Dairen I was not manifest in Vero cells which were poor substrates for Yaba virus replication, although the enhancing effect on Dis was
pronounced (Fig. 3). This finding also supports the idea that the rescuing activity may be separable from the inhibitory one. Poor conditions for Yaba virus replication may favour the plaque formation by Dis by minimizing the inhibitory activity of the rescuing virus.

The data shown in Fig. 5 indicate that an appreciable amount of Dis survived in JINET cells in a rescuable form at least for 5 days. Joklik et al. (1960) reported that heat-inactivated rabbitpox virus remained in a reactivable form in the mouse brain and HeLa cells at least for 3 days. Dis may survive in an eclipsed form in JINET cells because the rescuable Dis in living JINET cells was inactivated by disruption of the infected cells by sonication.

It remains to be decided whether the mechanism of the rescue phenomenon reported here is poxvirus-specific or the same as that of the enhancement by Yaba virus of plaque formation by certain DNA and RNA viruses (Tsuchiya & Tagaya, 1970; Tsuchiya & Tagaya, 1972a, b). The fact that Dis was rescued by Yaba virus suggests that Dis may be defective in a function common to Yaba virus and orthopoxviruses. The common function may be the one which is supposed to control the replication of orthopoxviruses at various stages of their replication cycle (Sambrook et al. 1965; Fenner & Sambrook, 1966). The factor whose activity evokes this function seems to be fairly resistant to u.v. because u.v.-inactivated Yaba virus possessed rescuing activity (Fig. 4). Therefore, the rescuing factor may be some virion component or early gene product of the rescuing virus.

REFERENCES


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