Genetic Analysis of Vaccinia Virus Lister Strain and Its Attenuated Mutant LC16m8: Production of Intermediate Variants by Homologous Recombination

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SUMMARY

We prepared vaccinia virus variants by introducing part of the HindIII D fragment of the DNA of the parental Lister (LO) strain (temperature-resistant and forming large plaques and pocks) into the attenuated LC16m8 strain (temperature-sensitive and forming small plaques and pocks) by the use of a homologous recombination technique in vivo. Special attention was paid to the HindIII D fragment, since this fragment has an extra XhoI site in LC16m8 which is absent from LO. After HindIII D of LO was introduced as a calcium phosphate precipitate into rabbit kidney (RK13) cells which had been infected with LC16m8, five virus variants (LOTC-1 to LOTC-5) forming much larger plaques than LC16m8 were cloned. In LOTC-2, LOTC-4 and LOTC-5, the introduction of at least part of HindIII D of LO into the corresponding HindIII D region of the LC16m8 genome was apparent as judged by the disappearance of the XhoI site, whereas variants LOTC-1 and LOTC-3 retained the site. The biological characteristics of all the LOTC variants were similar to each other. Their plaque size and pock size were similar to those of LO, whereas they were rather akin to LC16m8 with regard to temperature sensitivity and neurovirulence. The present results strongly suggested that part of the HindIII D fragment was involved in determining biological characteristics affecting plaque size and pock size, but had little influence on temperature sensitivity and neurovirulence.

Although smallpox has been eradicated from the world and vaccination has been discontinued, vaccinia viruses are now expected to play a new role: two groups (Panicali & Paoletti, 1982; Mackett et al., 1982; for review, see Mackett & Smith, 1986) have developed a method for the production and selection of infectious vaccinia virus recombinants expressing foreign genes, such as those coding for protective antigens of pathogenic viruses.

In order to use vaccinia virus as a vector for recombinant virus vaccines applicable to humans as well as to domestic animals, at least two conditions should be fulfilled. The first requirement is an attenuated character of the recombinant virus, especially low neurovirulence, since complications such as post-vaccinal encephalitis and encephalopathy have been rare but very serious problems in vaccination. The second is the ability of the recombinant vaccinia virus to express the foreign gene as well as to induce immune responses against the gene products.

A temperature-sensitive (ts) and low neurovirulence vaccinia virus variant, LC16m8, was isolated from the Lister (Elstree) strain (LO) by Hashizume et al. (1985). The mutant virus was used to inoculate more than 100000 children in Japan in 1974 and 1975, but no severe complications were reported. The LC16m8 virus has been authorized for use in Japan as a vaccine strain against smallpox for primary vaccination.
The LC16m8 strain is considered to be one of the safest vaccine strains ever used. However, because of the low level of infectivity in LC16m8 lesions (Hashizume et al., 1985) animals may not respond maximally to the non-vaccinia foreign protein. In the present experiments, we have produced hybrid virus variants possessing characteristics intermediate between LO and LC16m8, i.e. ts and low neurovirulence viruses forming large plaques and pocks. This result simultaneously suggested that the *HindIII* D fragment of the DNA of the Lister strain was at least partly responsible for determining the sizes of virus plaques and pocks.

Viruses used were WR, LO, LO-1 (a cloned derivative of LO), LC16m0, and LC16m8. They were grown on monolayers of rabbit kidney (RK13) cells. The origin and biological characteristics of LC16m0 and LC16m8 are reported in detail elsewhere (Hashizume et al., 1985). Purification of viruses was as described previously (Joklik, 1962).

DNA was extracted from purified virions essentially according to the method described by Sugimoto et al. (1985). Briefly, virions were lysed at room temperature overnight in 0.2 M-sodium phosphate buffer containing 6 M-urea, 0.01 M-EDTA, 1% sarkosyl NL 97, and 2% 2-mercaptoethanol. DNA was extracted twice with phenol:chloroform:i-soamyl alcohol (24:24:1) and then once with chloroform:i-soamyl alcohol (24:1), and passed through a Sephadex G-25 column (PD-10 column, Pharmacia). Purified vaccinia virus DNA was digested with *XhoI* endonuclease and the resultant DNA fragments were separated by agarose gel electrophoresis.

LO DNA was cleaved with endonuclease *SmaI* into two fragments, and the smaller DNA fragment was recovered from the gel by the method of Wieslander (1979). The recovered *SmaI* fragment was further digested with *HindIII*, and the resultant *HindIII* D fragment was cloned into pUC9 (pULOHD).

Monolayers of RK13 cells were infected with LC16m8 at an m.o.i. of 0.1 and 2 µg pULOHD DNA with 20 µg calf thymus DNA as carrier in the form of precipitates with calcium phosphate was transfected onto the cells. After 48 h incubation, viruses were recovered for isolation of virus variants.

Virus infectivity was assessed by plaque assay on RK13 cells or Vero cells (African green monkey kidney cells), or by pock formation on chorioallantoic membranes of 12-day-old embryonated eggs.

The one-step growth rate was investigated as follows. RK13 cell monolayers were prepared in a 25 cm² bottle, and virus was inoculated at an m.o.i. of 2. After adsorption of the virus at 37 °C for 60 min, 5 ml of culture medium was added. The virus titres were determined after 20 h cultivation.

The neurovirulence of the virus was assessed by two criteria, virus recovery and clinical symptoms in rabbits inoculated intracerebrally with viruses. Albino rabbits weighing 2.0 to 2.5 kg were inoculated intracerebrally with 10⁶.⁸ TCID₅₀ of the WR, LO, LC16m0, LC16m8 strains and the LOTC variants. The virus from the brain was titrated on day 3 or 6 after inoculation.

After transfection of LC16m8-infected RK13 cells with the LO *HindIII* D fragment, five virus variants, LOTC-1 to LOTC-5, forming apparently larger plaques than those of LC16m8 were purified by cloning three times. Virus DNA of each variant was digested with *XhoI*, and the digested DNA fragments were analysed by agarose gel electrophoresis (Fig. 1). The terminal F fragment of LO failed to form a clear band on the gel, owing to the heterogeneity of LO viruses (Wittek et al., 1978). The mobility of the F fragment of all five variants was identical (Fig. 1), and coincided with that of LC16m8 (data not shown) supporting the idea that all these variants were derived from LC16m8. There was an apparent difference in the electrophoretic patterns of fragments B of LC16m8 and LO. This was due to the *XhoI* site existing in the *HindIII D* fragment of LC16m8 alone: due to this additional site, a putative fragment of LC16m8 corresponding to the *XhoI* B fragment of LO is split into a smaller B fragment and a fragment nearly co-migrating with D (Sugimoto et al., 1985). From the electrophoretic patterns, it could be deduced that LOTC-1 and LOTC-3 were of the LC16m8 type while LOTC-2, LOTC-4 and LOTC-5 were derived from LO. It was concluded that the latter three variants were derived from LC16m8 by the incorporation of part of the LO *HindIII D* DNA fragment including the region corresponding to the *XhoI* site. From this experiment alone, it was not possible to
conclude whether or not part of the LO *Hind*III D fragment was also incorporated into LOTC-1 and LOTC-3.

The plaque size of all the LOTC variants was as large as that of LO (2.8 to 3.8 mm in mean diameter), and far larger than plaques of LC16m8 (1.7 mm) (Fig. 2). The same was true with the pock size: LOTC variants formed apparently larger pocks (3.5 to 3.8 mm in mean diameter) than LC16m8 (1.8 mm).

The infectivity of the viruses for RK13 cells and Vero cells was assessed by counting the number of plaques on monolayers of these cells 6 days after inoculation with various virus variants and strains (Table 1). LO and LC16m0 were very infective on both RK13 cells and Vero cells; the ratios of the titre assayed in RK13 cells to the titre in Vero cells for the two viruses were 0.22 and 1.0 respectively. On the other hand, LC16m8 was infective to RK13 cells but only poorly to Vero cells: the ratio was 360. LOTC variants were able to replicate well in both cell lines: the ratios were between 0.71 and 1.15. Therefore, as far as the plaque size, pock size and the infectivity to Vero cells were concerned, all the LOTC variants were of the LO type.

The growth rate was measured for each virus variant in single-step growth curves. As shown in Table 2, there was essentially no significant difference in the growth rate between LC16m8, LOTC variants and LO-1. These results indicated that in the viruses of the Lister strain, plaque size and pock size did not reflect virus growth rate within a cell. It seems likely that these indices would correlate with the ability of the virus to disseminate from cell to cell: viruses with higher disseminating ability would probably form larger plaques and pocks.
Fig. 2. LOTC variants and other viruses were adsorbed on RK13 cell monolayers, and cultivated in a medium containing agar at 35 °C for 4 days. A medium with neutral red was overlaid on day 4, and plaques were observed on day 5.

Table 1. Comparison of virus infectivity to RK13 cells and Vero cells*

<table>
<thead>
<tr>
<th>LOTC variant</th>
<th>-1</th>
<th>-2</th>
<th>-3</th>
<th>-4</th>
<th>-5</th>
<th>LO</th>
<th>LC16m0</th>
<th>LC16m8</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK13</td>
<td>$1.0 \times 10^7$</td>
<td>$3.0 \times 10^7$</td>
<td>$1.5 \times 10^7$</td>
<td>$1.5 \times 10^7$</td>
<td>$6.1 \times 10^6$</td>
<td>$2.0 \times 10^6$</td>
<td>$1.0 \times 10^7$</td>
<td>$6.0 \times 10^6$</td>
</tr>
<tr>
<td>Vero</td>
<td>$1.1 \times 10^7$</td>
<td>$3.4 \times 10^7$</td>
<td>$2.1 \times 10^7$</td>
<td>$1.7 \times 10^7$</td>
<td>$5.3 \times 10^6$</td>
<td>$9.2 \times 10^6$</td>
<td>$1.0 \times 10^7$</td>
<td>$1.7 \times 10^4$</td>
</tr>
<tr>
<td>Ratio (RK13/Vero)</td>
<td>0·91</td>
<td>0·88</td>
<td>0·71</td>
<td>0·88</td>
<td>1·15</td>
<td>0·22</td>
<td>1·00</td>
<td>360</td>
</tr>
</tbody>
</table>

* Serial tenfold dilutions of the viruses in 0·2 ml original solution (2·0 $\times 10^6$ to $3.0 \times 10^7$ p.f.u. per 0·2 ml as assayed in RK13 cells) were inoculated onto either RK13 cells or Vero cells. The number of plaques was determined 5 days after inoculation; virus titres were determined as p.f.u./0·2 ml.

Table 2. Single-step growth of LOTC variants

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC16m8</td>
<td>7·5†</td>
<td>7·7</td>
</tr>
<tr>
<td>LO-1</td>
<td>7·1</td>
<td>–</td>
</tr>
<tr>
<td>LOTC-1</td>
<td>7·3</td>
<td>7·1</td>
</tr>
<tr>
<td>LOTC-2</td>
<td>8·3</td>
<td>8·1</td>
</tr>
<tr>
<td>LOTC-3</td>
<td>7·9</td>
<td>7·9</td>
</tr>
<tr>
<td>LOTC-4</td>
<td>8·1</td>
<td>8·1</td>
</tr>
<tr>
<td>LOTC-5</td>
<td>7·9</td>
<td>8·1</td>
</tr>
</tbody>
</table>

* Viruses were adsorbed to RK13 cell monolayers at an m.o.i. of 2, and virus titres were determined 20 h after inoculation.
† Titres are expressed as logarithms.
Temperature sensitivity of viruses in primary rabbit kidney cells*

| LOTC variants | At 35 °C | At 40.8 °C | Ratio 

(35 °C/40.8 °C) |
|---|---|---|---|
| -1 | $4.8 \times 10^7$ | <10 | >$4.8 \times 10^5$
| -2 | $1.4 \times 10^7$ | <10 | >1.4$x 10^3$
| -3 | $9.0 \times 10^6$ | $10^2$ | 9$x 10^4$
| -4 | $6.4 \times 10^6$ | $10^2$ | >6.4$x 10^5$
| -5 | $2.3 \times 10^6$ | $10^2$ | 9.2$x 10^3$
| LO | $9 \times 10^6$ | $2.5 \times 10^2$ | 4.5$x 10^3$
| LC16m0 | $2.8 \times 10^6$ | $2 \times 10^5$ | >2.8$x 10^5$
| LC16m8 | $2.4 \times 10^6$ | <10 | >2.4$x 10^5$

* Plaque assay of the viruses was performed 3 days after virus inoculation in primary rabbit kidney cells at 35 °C and 40.8 °C.

† P.f.u./0.2 ml original solution; each point represents the mean of two samples.

Temperature sensitivity of the viruses was investigated by the use of monolayers of primary rabbit kidney (p-RK) cells at 35 °C and 40.8 °C. As expected, in the case of LO many plaques were formed at 40.8 °C as well as at 35 °C: the ratio of the titre at 35 °C to that at 40.8 °C was 45, whereas LC16m0 and LC16m8 formed plaques almost exclusively at 35 °C; the ratio was more than 100000 (Table 3). All the LOTC variants were shown to be temperature-sensitive; the ratio of the titre at 35 °C to that at 40.8 °C was between 92000 and 640000.

Rabbits were inoculated intracerebrally and the virus titres in the brain were determined on days 3 and 6 after inoculation. Signs of disease were also monitored. Viruses of higher titres, between 3.5 and 5.25 TCID_{50}/ml of 10% brain homogenate, were recovered on day 3 from brains inoculated with LOTC variants, LO or LC16m0 strains. On the other hand, virus recovery was low in the case of LC16m8 (1.8 TCID_{50}/ml). The brain inoculated with the WR strain showed a very high titre (7.3 TCID_{50}/ml). On day 6 the virus recovery from animals inoculated with LOTC variants, or LC16m0 or LC16m8 strains was much lower (between 0.5 and 1.4 TCID_{50}/ml) than the recovery from those inoculated with the LO or WR strains (3.6 and 6.4 TCID_{50}/ml respectively).

Two of three rabbits inoculated with WR displayed paralysis of the hind legs (the remaining one died), and one of four rabbits inoculated with LO displayed similar paralysis. On the other hand, all 14 rabbits inoculated with LOTC variants (each variant virus was inoculated into two to four rabbits) and all those inoculated either with LC16m0 (three rabbits) or LC16m8 (three rabbits) remained healthy, indistinguishable from uninoculated control rabbits.

The five vaccinia virus variants, LOTC-1 to LOTC-5, isolated in the present investigation revealed biological characteristics intermediate between LC16m8 and LO. The genomes of three of the variants, LOTC-2, LOTC-4 and LOTC-5, were very likely to contain part of the LO HindIII D DNA fragment as judged by the absence of the *XhoI* site marker there. As for the *XhoI*-positive LOTC-1 and LOTC-3, it was noticed that all of their biological characteristics showed them to be indistinguishable from the *XhoI*-negative variants. On the basis of these results, we would speculate that the *XhoI*-positive variants LOTC-1 and LOTC-3 may also contain part of the LO HindIII D region as the consequence of homologous recombination *in vivo* rather than having arisen through spontaneous mutation.

There was very good agreement between plaque size, pock size and sensitivity of Vero cells: LC16m8 alone showed low values in all these indices. As discussed above, the LOTC variants seemed to be recombinant viruses derived from LC16m8 by receiving part of the LO HindIII D DNA. These facts strongly suggested that HindIII D of the Lister strain was at least partly involved in the determination of plaque size, pock size and sensitivity of Vero cells. The putative gene(s) may be located near the *XhoI* site but probably do not include the site itself.

Temperature sensitivity and disease-producing potential were correlated and unrelated to the characteristics of the above-mentioned virus. It is likely that most of the genes responsible for the temperature sensitivity and disease-producing potential were located outside the HindIII D region of the genome.

Since the usefulness of vaccinia virus as the vector for expression of foreign antigen genes was discovered, many kinds of recombinant vaccinia viruses have been made (for review, see Mackett & Smith, 1986), in most cases from the WR strain. This strain possesses a strong ability...
Short communication

to infect and disseminate which is convenient for expressing foreign antigen genes and for inducing immunity against the antigen. It is, however, not practical to use WR as a live recombinant vaccine vector because of its high neurovirulence.

In order to make a practical vaccine, we attempted to express a recombinant vaccine by the use of LO, LC16m0 and LC16m8 as vectors, and confirmed that the recombinants derived from attenuated vaccinia virus retained the attenuated character (Morita et al., 1987). One problem in the use of, for example, LC16m8 as a vector is its poor ability to induce immune responses against the foreign antigen, probably due to its low ability to disseminate from cell to cell. In this regard, the LOTC variants would possess some advantageous characteristics as vectors, that is, relatively low neurovirulence with high disseminating capacity. Interesting prospects in the future would be to make a recombinant virus using LOTC variants as vectors, and to assess the applicability of the recombinant as a live vaccine.

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REFERENCES


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