Effect of In Vitro Adaptation of Marek's Disease Virus on Pock Induction on the Chorioallantoic Membrane of Embryonated Chicken Eggs

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Cell-associated preparations of several isolates of Marek's disease virus produced more pocks on the chorioallantoic membrane of embryonated chicken eggs than plaques in duck embryo fibroblasts, thus indicating that lesion response in eggs was more sensitive than cytopathic response in duck embryo fibroblasts for assaying low-passage Marek's disease virus. Adaptation of the virus to cell cultures by serial passages, however, substantially reduced its pock response so that the titer ratio (plaque-forming units in duck embryo fibroblasts/pock-forming units in eggs) of cell culture-adapted Marek's disease virus was 1 or higher. The decreased pock response could not be attributed to selection of preexisting virus variant(s) with low affinity for chorioallantoic membrane because cloned Marek's disease virus had a good pock response at low cell culture passage levels, but this response decreased as the virus was attenuated by serial cell culture passage.

Marek's disease (MD) of chickens is caused by a herpesvirus. MD virus (MDV) propagates well in avian cell cultures (11, 13), whereas mammalian cells (3, 13, 16) and mammals (15, 16) are refractory. Monolayer cultures of chicken kidney or duck embryo fibroblasts (DEF) cells are most commonly used to assay MDV. In these cultures, MDV induces cytopathic changes (plaques) typical of herpesviruses. MDV may also be assayed in embryonated chicken eggs. Infected embryos develop macroscopic lesions (pocks) on the chorioallantoic membrane (CAM) (B. M. Longenecker, F. Pazderka, and R. F. Ruth, submitted for publication; 2, 8, 9, 17) and other pathological changes (8). The pock response can be obtained by depositing the virus inoculum in various locations in the egg, although the intravenous route may be the most sensitive (Longenecker et al., submitted for publication).

Upon prolonged serial tissue culture passage, MDV loses pathogenicity for chickens but retains immunogenicity. Thus, tissue culture-attenuated virus has been successfully used in the field as a vaccine against pathogenic strains (6). The in vitro adaptation of MDV generally results in more rapid growth of the virus in cell cultures; the cytopathic effect appears faster and the virus replicates to a higher titer than the unadapted progenitor. Our objective in this study was to determine if tissue culture adaptation of MDV would affect its ability to induce pocks on the CAM.

The GA isolate of MDV (7) was cloned by Purchase et al. (12) and used at serial passage 19 in DEF. Another preparation of uncloned GA isolate (supplied by Lucy Lee) was used at serial passage 60 in DEF. Several preparations of cloned and uncloned JM isolates were used. Uncloned JM (18) was serially passed in chicken embryo fibroblasts (CEF) and DEF and was used at passage levels 56 and 104 (supplied by W. Okazaki and K. Nazerian). Clones 30 and 31 of JM were obtained from H. G. Purchase (12). Various passage levels of clone 102W of JM were supplied by R. L. Witter. This clone was obtained by three-step cloning of the JM virus (R. L. Witter, unpublished data) and was used at passage levels 13, 60, 110, and 120 in DEF. RPL-39 isolate originated from a field outbreak of MD in Georgia (10). The HN isolate, an apathogenic virus isolated from specific pathogen-free chickens by Zander et al. (21), was supplied by B. R. Cho (4) and was used at serial passage 7 in CEF. The cell-associated preparations of the FC126 isolate of herpesvirus of turkeys in CEF were used (19).

Viable cell-associated preparations of various isolates were kept frozen at −196 °C in sealed glass ampules. At the time of use, the ampules were quick thawed at 37 °C and immediately diluted with tissue culture medium. Tenfold serial dilutions were assayed simultaneously in DEF and in embryonated chicken eggs.

Duplicate cultures of 24-h-old secondary DEF cell cultures (20) were inoculated with each 10-
fold serial dilution of virus, using 0.1 ml of inoculum per culture. At 24 h after inoculation, cultures were overlayed with a medium 199 and F10 mixture containing 4% bovine serum and 1% agar. Additional quantities of agar overlay medium were added twice a week. Plaques were enumerated 12 to 14 days after inoculation.

Assays in embryonated eggs were done in fertile chicken eggs from outbred parents purchased from a commercial source (Rainbow Hatchery, St. Johns, Mich.). The MDV antibody status of these eggs was not assessed although they were assumed to possess this antibody because the donor hens were maintained under field conditions and had presumably survived natural infection with MDV. A portion (0.1 ml) of each virus dilution was inoculated intravenously into each of ten 11-day-old embryos. After 5 days of additional incubation at 37 C, surviving embryos were chilled overnight at 4 C and observed for pocks. Individual CAMs were carefully spread on a glass plate illuminated from below with a 22-W circular fluorescent tube. The entire CAM was enumerated for pocks.

For each virus preparation the plaque-forming units per ml in DEF were divided by plaque-forming units in eggs to obtain a titer ratio. Thus a titer ratio of less than 1 indicated that 1 ml of the virus preparation induced more pocks in embryos than plaques in DEF.

Among the 10 virus preparations shown in Table 1, the titer ratio varied from 0.06 to 4.71. In general all virus preparations that had not been adapted by serial tissue culture passage had a titer ratio of less than 1. Cell culture-adapted high-passage viruses had titer ratios that approached 1 (JM passage 56) or exceeded 1 (GA passage 60 and JM passage 104). The high-titer ratio was unrelated to the lack of pathogenicity because the nonpathogenic HN isolate and nonpathogenic herpesvirus of turkeys had ratios of 0.06 and 0.15, respectively. There was also no consistency between the presence or absence of A antigen and the titer ratio. Although the viruses with high titer ratio lacked A antigens, all viruses that lacked A antigen did not have high-titer ratios. For instance, JM clones 30 and 31 lacked A antigen yet had titer ratios of 0.44 and 0.74, respectively.

Thus the only consistent characteristic of the viruses with high-titer ratios was adaptation by serial cell culture passage. We postulated that in vitro adaptation may modify titer ratio by one of two ways: (i) from a mixture of variants present in the virus stock, selection occurs in serial cell culture passage, and the selected variants are nonpathogenic and have special affinity for cell cultures but grow poorly in embryos; or (ii) virus mutants during continued propagation in cell cultures and the mutation renders the virus nonpathogenic and deficient in its growth in embryos.

To rule out possibility (i), we examined clone-

<table>
<thead>
<tr>
<th>Virus</th>
<th>Isolate</th>
<th>Cell culture passage</th>
<th>Type of cell culture</th>
<th>Biological characteristics</th>
<th>Titer (PFU/ ml × 10^6)</th>
<th>Titer ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pathogenicity for chickens</td>
<td>Tissue tropism</td>
<td>A antigen production</td>
</tr>
<tr>
<td>MDV</td>
<td>GA</td>
<td>19</td>
<td>DEF</td>
<td>+</td>
<td>Viscerotrophic</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>DEF</td>
<td></td>
<td>-</td>
<td>NA^c</td>
<td>ND^d</td>
</tr>
<tr>
<td></td>
<td>JM (un-cloned)</td>
<td>18</td>
<td>DEF</td>
<td>+</td>
<td>Neurotropic</td>
<td>+</td>
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<tr>
<td></td>
<td>56</td>
<td>DEF</td>
<td>104</td>
<td>-</td>
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<td>-</td>
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<tr>
<td></td>
<td>JM clone 30</td>
<td>15</td>
<td>CEF + DEF</td>
<td>+</td>
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<td>-</td>
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<tr>
<td></td>
<td>JM clone 31</td>
<td>15</td>
<td>CEF + DEF</td>
<td>+</td>
<td>Neurotropic</td>
<td>-</td>
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<td></td>
<td>RPL-39</td>
<td>20</td>
<td>CEF + DEF</td>
<td>+</td>
<td>Viscerotrophic</td>
<td>+</td>
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<tr>
<td></td>
<td>HN</td>
<td>7</td>
<td>CEF</td>
<td>-</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>HVT^a</td>
<td>FC126</td>
<td>12</td>
<td>DEF</td>
<td>-</td>
<td>NA</td>
<td>+</td>
</tr>
</tbody>
</table>

^a PFU, Plaque-forming unit.  
^b Titer in DEF/titer in ovo.  
^c NA, Not applicable.  
^d ND, Not determined.  
^e W. Okazaki, unpublished data.  
^f HVT, Herpesvirus of turkeys.
purified MDV at various cell culture passage levels. Thus, clone 102W of the JM isolate, after three-step cloning, and presumably composed of progeny of a single virion, was simultaneously assayed in DEF and in embryonated chicken eggs at cell culture passage levels 13, 60, 110, and 120. Passage 13 of clone 102W is highly pathogenic for chickens, and preliminary data (Witter, unpublished data) indicate that higher passages are nonpathogenic. The results (Table 2) indicated that whereas passage 13 had a titer ratio of 0.25 or lower, this ratio had risen to 3.5 and 2.67 by cell culture passage level 60 in experiments 1 and 2, respectively. Passages 110 and 120 also had high-titer ratios. Although titer ratios varied somewhat in the two separate experiments, the consistency in the change of titer ratios from low to high with cell culture passage levels was apparent.

This study indicated that adaptation to cell cultures of GA and JM isolates of MDV by serial passage adversely affected their ability to induce pocks on the CAM of embryonated chicken eggs. Repeated serial passage of MDV in cell cultures results in loss of A antigen and renders the virus nonpathogenic for chickens. However, reduced ability of MDV to induce pocks on CAM could not be correlated with lack of pathogenicity for birds or with the presence or absence of A antigen. The naturally apathogenic HN isolate and the pathogenic clone of JM (clones 30 and 31) isolate, both producing A antigen, propagated to a higher titer in embryos than in DEF.

Our results contrast with those of Biggs and Milne (2) and Longenecker et al. (submitted for publication), who reported no obvious effect of attenuation of MDV on pock induction in the CAM. The reason for this discrepancy is not clear, although several factors such as the type of MDV isolate and conditions of in vitro attenuation and chicken embryo assay may influence response.

The mechanism by which tissue culture adaptation affects pock induction is not known. The change in titer ratio may be due to the emergence of new mutants or may simply reflect an increase in plating efficiency in cell cultures of the attenuated virus. The possibility that prolonged in vitro propagation resulted in selection of preexisting virus variant(s) with low affinity for embryonated chicken eggs was ruled out because a cloned preparation of MDV had a good pock response at low passage levels, but this response decreased as the virus was attenuated by serial cell culture passage.

The relationship between minimum infectious dose in chickens and plaque-forming units in cell culture of cell culture-propagated MDV has been variable. For instance, in a few studies reviewed by us, the minimum infectious dose values per milliliter of virus preparations were either higher (5, 14), about equal (20), or lower (1) than the plaque-forming values. In our study, a titer ratio of less than 1 was obtained with all low-passage isolates of MDV tested, indicating that 1 pock-forming dose in embryos would be substantially lower than 1 plaque-forming dose in DEF and may be equivalent to or lower than 1 chick infectious dose. Thus, pock response must be considered a highly sensitive method of assaying unattenuated cell culture-propagated MDV.

We are grateful to H. G. Purchase, R. L. Witter, W. Okazaki, Lucy Lee, K. Nazerian, B. R. Cho, and D. V. Zander for providing the viruses.

**LITERATURE CITED**


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**Table 2. Effect of cell culture adaptation of a cloned preparation (clone 102W) of MDV on its titer in DEF and in embryonated chicken eggs**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Tissue culture passage (in DEF)</th>
<th>Titer (PFU/ml x 10^a)</th>
<th>Titer ratio^b</th>
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<tr>
<td></td>
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<td>DEF</td>
<td>In ovo</td>
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<tr>
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<tr>
<td></td>
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<td>1.37</td>
<td>0.34</td>
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<tr>
<td></td>
<td>120</td>
<td>1.47</td>
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</tr>
<tr>
<td>2</td>
<td>13</td>
<td>.16</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>110</td>
<td>.90</td>
<td>0.13</td>
</tr>
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</table>

^a PFU, Plaque-forming unit.

^b Titer in DEF/titer in ovo.