The Attenuation, with Loss of Oncogenicity, of the Herpes-type Virus of Marek’s Disease (Strain HPRS-16) on Passage in Cell Culture

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SUMMARY
The herpes-type virus of Marek’s disease showed a gradual increase in the rate of development of cytopathic effect on passage in cell culture. By the 60th passage macroscopic plaques were produced after 6 days under fluid overlay, but no cell-free virus could be recovered after filtration of culture medium.

A loss of pathogenicity for chicks was noticed in the virus after 33 passages in cell culture. Furthermore, an antigenic change occurred between the 20th and 30th passage which was characterized by the loss of an antigen which could normally be found in the supernatant medium of cultures infected with low passage virus.

The possible origin of the attenuated virus is discussed.

INTRODUCTION
Previous investigations have provided strong evidence that a herpes-type virus is the cause of Marek’s disease (Churchill & Biggs, 1967; Solomon et al. 1968; Nazerian et al. 1968; Churchill & Biggs, 1968; Biggs et al. 1969).

The HPRS-16 strain of Marek’s disease virus remained cell-associated in chicken kidney cell cultures. It was found that the infectivity of such cells was destroyed when they were disrupted. The transfer of infection from cell to cell in monolayers was not inhibited by antiserum (Churchill, 1968). It was therefore postulated that the transfer of virus infection from cell to cell in culture was not mediated by released cell-free virus but was due to an undetermined mechanism requiring cell contact. Using infected chicken kidney cells as an antigen, precipitating antibodies were shown to develop in chickens following experimental infection with Marek’s disease (Chubb & Churchill, 1968). A comparison of isolates of both classical and acute Marek’s disease showed that the line of precipitation demonstrated was common to all, no antigenic differences being detected.

The object of the present study was to see whether continuous passage in cell culture might induce any changes in the properties of the virus.
METHODS

Experimental chickens. Houghton Poultry Research Station strain of Rhode Island Red chickens (HPRS-RIR) (Biggs & Payne, 1967) were used to provide the kidney cell cultures used up to the 30th tissue culture passage of the virus. Thereafter, Sykes’s line B of Rhode Island Red* (Biggs, Thorpe & Payne, 1968) were used for cell culture. This line shows an incidence of 100% of individuals genetically resistant to avian leucosis viruses of the A and B subgroups. In addition 50% are also resistant to subgroup C (Biggs & Payne, 1967; personal communication). These birds were chosen to minimize the possibility of contamination of cultures with leucosis viruses and because their cultures showed a uniform sensitivity to the herpes-type virus.

Cell culture. Kidney tissue was trypsinized using a magnetic stirrer at 37° and cultures prepared as described elsewhere (Churchill, 1968).

Virus. Virus isolated from the tumours of HPRS-RIR chickens at the 25th chick to chick passage of the HPRS-16 strain (Biggs et al. 1965; Purchase & Biggs, 1967) of acute Marek’s disease was used throughout the experiments. The virus isolation, passage and assay techniques in cell culture have been previously described (Churchill, 1968).

Infectivity assay in chicks. The short-term assay technique of Biggs & Payne (1967) was used and the titres were calculated by the method of Reed & Muench (1938).

The precipitation test. The agar gel for the double-diffusion precipitation test was made up to the following formula: sodium chloride, 4 g.; disodium hydrogen phosphate, 3.45 g.; potassium dihydrogen phosphate, 0.355 g.; Ionagar (Oxoid), 1.0 g.; sodium azide, 1.0 g.; and deionized water, 80 ml. Gel was poured on glass plates to a depth of approximately 3 mm. Wells 6 mm. in diameter were cut with a centre-to-centre distance of 9 mm. After the addition of antigens and antiserum the plates were allowed to stand for 48 hr at room temperature in a sealed box before examination.

Antiserum. The antiserum used to identify antigens in the gel diffusion test was collected from 2 cockerels that were hyperimmunized by the intramuscular inoculation of a suspension of disrupted infected cultured chicken kidney cells emulsified with Freund’s complete adjuvant. A course of six inoculations at 2 to 3-week intervals was given. The antiserum gave no reaction against antigens prepared from uninfected cultured chicken kidney cells, but did react with calf serum proteins, a constituent of the medium used for the culture of chicken kidney cells. Lines of precipitation due to the presence of calf serum protein in the cultured cell antigens were not found if one volume of calf serum was added to nine volumes of the hyperimmune cockerel serum before use in the test.

Precipitating antigens. Antigens consisting of disrupted cell suspension were prepared as previously described (Chubb & Churchill, 1968).

RESULTS

Plaque size

When low-passage HPRS-16 virus-infected cells were assayed it was noticed that microplaques of varying size were produced. After incubation for 7 days some infected centres were represented by tight clusters of rounded cells, while others occurred in

* Kindly supplied by F. and G. Sykes Ltd.
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the form of distinct holes, or microplaques, in the cell sheet. After 14 days, nearly all
the infected centres had developed into microplaques. The typical intranuclear inclusions (Churchill & Biggs, 1967; Churchill, 1968) could be found at 7 days associated
with both types of infected centre.

During the continuous passage of virus in cell culture a gradual increase in the rate
of development of the cytopathic effect in chicken kidney cells was noticed, such that
the period between each passage was reduced from 7 to 5 days and finally to 3 days
by the 60th passage. This increased rate of development of cytopathic effect was also
manifested by an increase in the plaque size produced. The plaques produced by low-
passage virus remained microscopic at 9 days, and even by 14 days only a proportion
had become macroscopic (Plate 1a). In contrast, by the 60th passage all infected
centres gave rise to macroscopic plaques which developed in 6 days and were about
1.5 mm. across by the 9th day (Plate 1b). It can be seen that the high-passage-level
virus still produced some variation in plaque size; nevertheless, this was less than that
produced by the low-passage virus. The basic details of the cytopathic effect including
the production of intranuclear inclusions remained unaltered although an increased
incidence of polykaryocytes was noticed.

Absence of cell-free virus

The supernatant medium from heavily infected cultures in which more than 25 %
of the cells were estimated to be pathological was repeatedly tested for the presence
of cell-free virus, at various passage levels up to the 80th. No infectivity was ever recovered
in this series of experiments after such supernatant media had been passed through
Millipore filters of 1.2 μm. pore size. A generalized cytopathic effect occurred only
where the cultures were treated with infected cell inocula of high enough infectivity
to cause the primary plaques to coalesce.

Pathogenicity of cell culture passaged virus for chicks

During the course of the first 61 passages in cell culture the virus was tested for its
pathogenicity for day-old HPRS-RIR chicks at various passage levels (see Tables 1, 2).
In most cases this was assessed on the production of gross and/or histological lesions
in a 3-week experimental period (Table 1). In some experiments a longer experimental
period was allowed; in these the results were assessed on mortality (Table 2) supported
by histology where gross lesions could not be found. It will be seen from the tables
that the pathogenicity of infected cultured cells for chicks was clearly demonstrated
up to the 20th passage of the virus, but by the 33rd passage this pathogenicity was
apparently lost. The non-pathogenic nature of the virus was maintained thereafter up
to the 61st passage, the highest level tested.

Comparative assays of infected cultured cell preparations in chicks and in cell
cultures showed that the ID₅₀ for chicks was equal to between 3 and 20 microplaque
forming units (mp.f.u.) during the first passages (Table 3). However, at the 33rd
passage a dose of 10⁴ mp.f.u. proved to be completely non-pathogenic in the 3-week
experimental period used (Table 1). Furthermore, at the 39th passage a dose of 10⁴.⁴⁶
mp.f.u. administered intraperitoneally to 14-day-old chicks induced no cases of
Marek’s disease in the 23-week observation period (Table 2).
Influence of cell genotype

Because the strain of chicken used for cell cultures had been changed at the 30th virus passage (see Methods), it was considered necessary to test whether the change in pathogenicity that occurred in the virus by the 33rd passage was in some way connected with this. Stored virus-infected cells from the 9th passage in HPRS-RIR chicken kidney cells were used to initiate three consecutive passages in both HPRS-RIR cells and Sykes line B cells in parallel. At the end of this time the virus harvested from each series of passages and the chick pathogenicity was compared with that of the 9th passage. Groups of 18 chicks were inoculated with approximately $10^4$ mp.f.u. per chick. Each of the three inoculated groups showed an incidence of Marek's disease of more than 85% when assessed at 3 weeks by the short-term assay procedure, while 18 control chicks remained negative. These results indicate that the change in source of cells used for culture was not responsible for the change in pathogenicity for chicks of the virus.

Antigenic change

Both chicken-kidney-cell extracts and supernatant culture fluid from heavily infected monolayers were shown to contain antigens that produced a line of precipitation when reacted against sera from Marek's-disease-infected chickens in the agar-gel double-diffusion precipitation test (Chubb & Churchill, 1968). Using sera from hyper-immunized cockerels, up to six lines of precipitation have been seen under optimum

### Table 1. The pathogenicity for chicks in a 3-week experimental period of the herpes-type virus of Marek's disease (strain HPRS-16) after passage in cell culture

<table>
<thead>
<tr>
<th>Passage level</th>
<th>Dose (mp.f.u.)</th>
<th>Number inoculated</th>
<th>Gross lesions†</th>
<th>Histological lesions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>17,250</td>
<td>18</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>12</td>
<td>5,600</td>
<td>17</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>14</td>
<td>18</td>
<td>9</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>3,750</td>
<td>14</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>20</td>
<td>65</td>
<td>8</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>33</td>
<td>10,800</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>39</td>
<td>160</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>42</td>
<td>5,650</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The number of mp.f.u. each chick received by intraperitoneal inoculation at 1 day of age.
† The number with lesions at autopsy when killed 3 weeks after inoculation.

### Table 2. The pathogenicity for chicks over an extended experimental period of Marek's disease virus (strain HPRS-16) after passage in cell culture

<table>
<thead>
<tr>
<th>Passage level</th>
<th>Age at inoculation (days)</th>
<th>Dose (mp.f.u.)</th>
<th>Experimental period (weeks)</th>
<th>Number inoculated</th>
<th>Marek's disease deaths†</th>
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</thead>
<tbody>
<tr>
<td>15</td>
<td>1</td>
<td>800</td>
<td>10</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>39</td>
<td>1</td>
<td>160</td>
<td>10</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>39</td>
<td>14</td>
<td>1,026</td>
<td>23</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>61</td>
<td>1</td>
<td>11,500</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

* The number of mp.f.u. inoculated by the intraperitoneal route.
† Number of birds which died with either gross or microscopic lesions of Marek's disease.
(a) CK monolayer showing microplaques 4 days after infection with the 7th tissue culture passage of MD HTV strain HPK-16 (stained with May–Grunwald–Giemsa, ×1.5).

(b) CK monolayer showing the plaques produced 6 days after infection with the 61st passage of HPK-16 (stained with May–Grunwald–Giemsa, ×1.5).
(a) Precipitation lines produced by the following antigens against hyperimmune antiserum no. 1 (centre well). Well no. 1: supernatant from the 10th tissue culture passage of HPRS-16 (concentrated × 50); 2: uninfected tissue culture cell antigen; 3: as well 1; 4: 87th tissue culture passage of HPRS-16 (cell antigen); 5: 10th tissue culture passage of HPRS-16 (cell antigen); 6: tissue culture maintenance medium. Complete and unused.

(b) Centre well—hyperimmune antiserum no. 1. Well no. 1: 87th tissue culture passage of HPRS-16 (cell antigen); 2: 11th tissue culture passage of HPRS-16 (cell antigen); 3: supernatant from the 10th tissue culture passage of HPRS-16 (concentrated × 50); 4: as well 2; 5: as well 3; 6: as well 2.

(c) Centre well—hyperimmune antiserum no. 1. Well no. 1: 87th tissue culture passage of HPRS-16 (extracted cell antigen); 2: 10th tissue culture passage of HPRS-16 (extracted cell antigen); 3: 10th tissue culture passage of HPRS-16 (supernatant concentrated × 50); 4: as well 1; 5: as well 3; 6: 8th tissue culture passage of HPRS-16 (extracted cell antigen).

(d) As for (c), except that centre well contains hyperimmune antiserum no. 2.

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Conditions. However, under the conditions of our system only the strongest three of these can be regularly identified and therefore our study is limited to these only.

The strongest line of precipitation produced by antigens extracted from infected cultured cells represented an antigen that was also found released into the supernatant culture medium. For convenience, this antigen has been referred to as antigen A and was the only one detectable in supernatant medium even after a 50 times concentration by forced dialysis. Using infected cell extracts at the concentration described in the Methods section, two other strong lines of precipitation could be detected. These have been referred to as antigens B and C.

Table 3. Comparative assays of Marek's disease virus (strain HPRS-16) in cell culture and in chicks

<table>
<thead>
<tr>
<th>Passage level</th>
<th>Titre mp.f.u./ml.*</th>
<th>Titre ID 50/ml.†</th>
<th>Ratio mp.f.u./ID 50</th>
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</thead>
<tbody>
<tr>
<td>7</td>
<td>(10^{1.2})</td>
<td>(10^{3.8})</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>(10^{3.8})</td>
<td>(10^{1.2})</td>
<td>6.5</td>
</tr>
<tr>
<td>14</td>
<td>(10^{3.4})</td>
<td>(10^{3.8})</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>(10^{3.7})</td>
<td>(10^{3.4})</td>
<td>22</td>
</tr>
</tbody>
</table>

* Titre in cell culture of the suspension of infected cells harvested from the passage level indicated.
† Results of an infectivity assay by the intraperitoneal inoculation of 1-day-old IRK chicks.

Table 4. The pathogenicity for chicks of virus obtained by a limiting dilution technique from the 15th cell culture passage of Marek's disease virus (strain HPRS-16)

<table>
<thead>
<tr>
<th>Limiting dilution isolate (no.)</th>
<th>Sub-type</th>
<th>Dose per chick in mp.f.u.</th>
<th>Gross lesions at autopsy</th>
<th>Histological lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mpA+</td>
<td>10,464</td>
<td>6/14</td>
<td>13/14</td>
</tr>
<tr>
<td>2</td>
<td>MPA-</td>
<td>12,960</td>
<td>0/14</td>
<td>2/14</td>
</tr>
<tr>
<td>3</td>
<td>MPA-</td>
<td>4,480</td>
<td>0/14</td>
<td>0/14</td>
</tr>
<tr>
<td>1 and 2 mixed</td>
<td>mpA+ and MPA-</td>
<td>7,712</td>
<td>6/14</td>
<td>10/14</td>
</tr>
<tr>
<td>Control cells</td>
<td></td>
<td>-</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
<td>Parent*</td>
<td>mpA+</td>
<td>3,776</td>
<td>10/14</td>
<td>13/14</td>
</tr>
</tbody>
</table>

* Parent virus = the original 15th cell culture passage which was used to prepare the limiting dilution.

During the course of passaging the HPRS-16 strain of Marek's disease virus in chicken kidney cell culture antigen A disappeared. Infected cell antigens and the supernatant medium tested at various virus passage levels showed a gradual weakening of the A antigen after the 20th passage, with its complete disappearance by the 30th. Antigens B and C were found associated with infected cell extracts right through the series of passages up to the 87th passage, the highest level tested. Plate 2a to 2d illustrates the patterns of precipitation lines produced with low- and high-passage level virus antigens. The two antigenic types thus observed were a microplaque producer with the A antigen and a macroplaque producer without the A antigen. These were designated in an abbreviated form as mpA+ and MPA- respectively.

Virus passage from the 15th to the 30th passage was repeated by thawing cells infected with the 15th passage of the virus from storage in dimethyl sulphoxide in a liquid nitrogen refrigerator. During the course of this repeated passage series the A antigen was again observed to diminish and disappear.
Virus isolations from the limiting dilution

Attempts were made to isolate mpA+ or MPA− virus from preserved stocks of virus harvested just before the observed change by the limiting dilution technique. A series of fourfold dilutions was made of the 15th passage virus. Each dilution was inoculated on to 20 plates, and thereafter each plate was individually passaged 8 times during 6 weeks in culture. At the end of this time six out of 20 plates inoculated with \(10^{-5.2}\) limiting dilution of the frozen cell stock showed virus cytopathic effect, while the remaining 14 and controls were negative. Of the six virus lots thus isolated, two were found to be mpA+ and four were MPA−. Two MPA− and one mpA+ viruses thus isolated were selected for a chick-pathogenicity test. One group of chicks was also inoculated with a mixture of MPA− and mpA+-infected cells to determine whether, in the event of the MPA− virus proving non-pathogenic, it would modify the pathogenicity of an mpA+ virus. Three weeks after inoculation it appeared that the mpA+ virus was highly pathogenic, while the MPA− viruses were of little or no pathogenicity (Table 4). When inoculated simultaneously, the MPA− virus did not interfere with the pathogenicity of the mpA+ virus.

Interference in cell culture with the production of macroscopic plaques

In order to determine whether MPA− virus was able to produce macroscopic plaques in the presence of excess mpA+ virus, a batch of chicken kidney monolayers was divided into three groups. Groups 1 and 2 were infected with different levels of mpA+ virus \((10^{3.9} \text{ mp.f.u. and } 10^{3.4} \text{ mp.f.u. per plate})\) while group 3 was left untreated. Immediately following this, a stock of MPA− virus was assayed on each of the three groups of monolayers by counting the macroscopic plaques produced at 6 days after inoculation. The titration results from these three assays using the same MPA− virus dilutions were \(10^{3.55}, 10^{3.62}\) and \(10^{3.67}\) macroscopic plaque-forming units per 0.2 ml. The ratio of mpA+ to MPA− virus on the plates counted for the titre calculations was approximately 170:1.

These results indicate that when inoculated simultaneously an excess of mpA+ virus does not interfere with the production of macroscopic plaques by MPA− virus.

DISCUSSION

The results reported here demonstrate that during the passage of the HPRS-16 strain of herpes-type virus in cell culture, certain characteristics were altered. The basic details of cytopathic effect and virus structure (Churchill & Biggs, 1967; Churchill, 1968) remained unaltered, while the rate of development of cytopathic effect, antigenic constitution and pathogenicity all showed changes. We consider that the explanation for these changes may lie in one of the following four hypotheses: (i) that a contaminant was introduced in the form of another cell-associated herpes-type virus, which was itself non-pathogenic for chicks, and which outgrew the original isolate; (ii) that the original isolate was a heterogenous collection of two or more herpes-type virus variants. One of these which was typified by the new properties was favoured by the \textit{in vitro} culture conditions and was thus selected during passage; (iii) that an unidentified infectious agent distinct from the herpes-type virus was responsible for both production of the A antigen in cell culture and the induction of Marek’s disease.
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in inoculated chicks, and that during the passage of the HPRS-16 strain in cell culture
the unidentified agent was lost while the herpes-type virus has survived; (iv) that a
variant has arisen by mutation during in vitro passage.

During the serial passage of the virus uninoculated cell culture controls from all
batches of cell culture were examined for evidence of cytopathic effect. On two
occasions cytopathic effect was found in controls after the batch had been put into
use. In these cases, the relevant passage was discarded and resumed from the last
stored frozen stock of infected cells. However, continuously passaged controls were
not maintained throughout the whole series of passages, so that a latent infection of a
type that would only be revealed by serial blind passage would not have been
detected. Two isolates from infected control cultures have been characterized and
were found to be of the mpA + type and to be pathogenic for chicks (Churchill &
Biggs, 1968). No virus of the MPA- type has yet been recovered from control cultures.

The only available evidence against hypothesis (ii) is that low passage HRPS-16 virus
did not produce any macroscopic plaques in 6 days incubation, even when 10^4 mp.f.u.
were inoculated per plate. This observation suggests that if MPA- virus was present
in the original isolate it formed less than 1/10,000th part. Furthermore, the results of
the interference experiment indicate that our failure to detect macroscopic plaques
in the early passages could not be accounted for by interference of the mpA+ virus
with the MPA-. Nevertheless, hypothesis (ii) cannot be entirely ruled out. The third
hypothesis given has been discussed by Churchill & Biggs (1968) and Biggs et al
(1969), when presenting evidence to negate the concept that the herpes-type virus is
a separate and independent infectious agent from the causal agent of Marek's disease.

The fourth hypothesis appears to us to be the most acceptable explanation at present.
It suggests that the virus has become modified by the emergence of a mutant (MPA-)
during passage in cell culture and that this mutant has been favoured by its increased
rate of spread in cell culture until it has outgrown the parent virus, as indicated by
the complete apathogenicity for chicks of large doses of the high passage level virus.

We wish to thank Mr G. Carrington and Mr S. Edler for their able technical assistance.

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