Reversion to Virulence of Attenuated Canine Distemper Virus In Vivo and In Vitro

By MAX J. G. APPEL

James A. Baker Institute for Animal Health, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

(Accepted 9 June 1978)

SUMMARY

Reversion to virulence of dog kidney cell attenuated canine distemper virus (CDV), Rockborn strain, was demonstrated after serial passage in dogs. The same strain also reverted to virulence after serial passage in canine macrophage cultures. Criteria for virulence in dogs included clinical signs, weight loss, elevated body temperatures, reduced total lymphocyte counts, impaired blastogenesis responses of blood lymphocytes, appearance of virus antigen in epithelial cells, the character of neutralizing antibody responses and the capability of re-isolated virus to grow in canine macrophage cultures.

INTRODUCTION

Virus pathogenicity of a particular strain is determined by challenge of experimental animals. While a virulent virus would produce overt disease, attenuated or avirulent virus should not, when the same amount of virus is inoculated. The degree of virulence may vary greatly and may be modulated by host factors, such as age, genetic constitution, state of pregnancy, state of immune competence, or the host-response to a particular virus. Although the search for virus properties linked to virulence has been extensive, only a few phenotypic markers have been identified. The most common in vitro markers for virulent or avirulent virus are the type of permissive tissue culture (TC), nature and size of plaques in TC and temperature sensitivity (Kantoch, 1978). If growth of virus is restricted in certain cell populations, or at normal or elevated body temperature, it generally becomes avirulent.

Virulent canine distemper virus replicates readily in dog macrophage cultures (Appel & Jones, 1967; Poste, 1971), but adaptation is required for its in vitro growth in epithelial cells (Rockborn, 1958). Once adapted, however, virulence is rapidly lost (Rockborn, 1959; Harrison et al. 1968). Dog kidney cell-attenuated virus (Rockborn strain) replicates well in several cell lines, but, initial growth in dog lung macrophage cultures is limited (M. Appel, unpublished data). The basis for this observation is not known. Because adaptation of virulent CDV to epithelial cells and attenuated CDV to macrophages occurs within a few passages, it must be assumed that either a mixed population of virus particles with different characteristics is present in virulent and attenuated CDV strains or cellular membrane maturation of virus influences virulence. In any event, virulence in CDV appears to be linked to the capability of virus to infect and replicate in dog macrophages. We tested this hypothesis by serially passaging attenuated CDV in dogs, and in dog macrophage cultures in vitro.
METHODS

Experimental animals. Three-month-old specific pathogen free (SPF) beagle dogs from the Institute's colony were used for this study. Non-infected dogs were kept separate from the infected animals in isolation units. Dogs were fed with standard commerical rations.

Virus. The dog kidney (DK) cell-attenuated Rockborn strain of CDV (CDV-RO; Rockborn, 1959) at approx. the 40th passage level in DK cells was used for animal inoculations and serial passage studies. The Vero cell-adapted Onderstepoort strain of CDV (CDV-Ond; Appel & Robson, 1973) was used for serum neutralization tests.

Tissue culture. Primary DK cells were used for propagation and titration of CDV-RO. Cells were prepared from 2- to 4-week-old SPF pups and were grown in vitro in MEM with Earle's salt and non-essential amino acids (GIBCO, Grand Island, N.Y.). L-Glutamine (2 mM), 10% foetal calf serum (FCS) and antibiotics were added for growth medium. FCS was reduced to 2% for maintenance medium.

Primary dog lung macrophage (DLM) cultures were prepared from the DK cell donors for growth and titration of virulent CDV as described previously (Appel & Jones, 1967) with minor modifications. Briefly, lungs were minced with scissors and stirred in medium 199 (GIBCO, Grand Island, N.Y.) for 1 h at 20 °C for mechanical separation of macrophages. They were maintained in medium 199 + 20% FCS at 37 °C in Leighton tubes, or in MEM with 20% FCS at 37 °C in microplates in an atmosphere of 5% CO₂ and 95% air. FCS which was non-toxic to macrophages and free of non-specific virus inhibitors was selected.

Virus titrations. Because of poor plaquing properties of CDV-RO in DK cells, serial tenfold dilutions of virus suspensions in MEM each were inoculated in 0.1 ml amounts into four Leighton tubes seeded with primary DK cells. Media changes were made three times per week. Ten days post-infection (p.i.), titration endpoints were determined by examinations of acetone fixed coverslips using a direct immunofluorescence (IF) method.

Titrations for virulent virus were made in plastic microplates (IS-FP-96 TC, Linbro Chemical Co., Inc., New Haven, Conn., U.S.A.). Threefold dilutions of virus suspensions were made with microdiluters in 0.1 ml MEM + 20% FCS per well; approx. 10⁶ macrophages in 0.05 ml MEM + 20% FCS were added to each well. Plates were covered with sterile gauze and plastic lids and were incubated for 5 days at 37 °C in an atmosphere of 5% CO₂, 95% air. At that time, titration endpoints were determined by the formation of syncytia, the criteria for CDV replication (Appel & Jones, 1967). Titration endpoints were retested in macrophages in Leighton tubes. Five days after infection, coverslips were fixed in acetone and examined by IF for presence of virus antigen.

Adaptation of attenuated virus to DLM cultures. For the adaptation of CDV-RO to DLM cultures, CDV-RO infected DK cells were removed from a tissue culture flask with versene-trypsin and mixed with freshly prepared DLM cells. After 3 days, when syncytial formation of DLM cells became apparent, cultures were frozen and thawed. After removal of cellular debris, undiluted fluid was inoculated into freshly prepared DLM cultures. Ten serial passages were then made in DLM cells.

Virus neutralization. Virus-neutralizing antibody was titrated in Vero cell culture using CDV-Ond as test virus. All tests were performed in microplates as described (Appel & Robson, 1973).

Mitogen blastogenesis of blood lymphocytes. Stimulation with phytohaemagglutinin (PHA) of canine blood lymphocytes was done as described previously (Schultz, 1977). Briefly, 2 ml of heparinized blood were overlaid onto 3 ml of Ficoll-Hypaque (Pharmacia
Reversion to virulence of attenuated virus

Fine Chemicals, Piscataway, N.J.) in a 12 × 75 mm plastic vial (no. 2054 – Falcon, Oxnard, Calif.), then centrifuged at 527 g for 15 min. The lymphocyte rich upper layer was resuspended in medium RPMI-1640 (GIBCO, Grand Island, N.Y.) with 20% FCS and adjusted to 4 × 10^6 lymphocytes/ml. This suspension was placed in 0.05 ml amounts in each of 6 wells in a 96-well microplate. A PHA suspension was prepared as described previously (Brooke, 1962) and titrated and diluted for optimal stimulation of dog lymphocytes. The dilution was added in 0.05 ml amounts to 3 wells, and 3 wells were kept as controls. After 3 days of incubation at 39 °C in 5% CO₂, 0.1 ml of RPMI-1640 medium containing 1 µCi ^3H-thymidine was added to each well (sp. act. 20 Ci/mm). Eighteen h later, cells were harvested on filter pads in an automated cell harvester (Mash II, Microbiological Associates, Bethesda, Md.). Filter pads were dried at 60 °C for 15 min, placed in 5 ml of scintillator solution (Eastman Kodak, Rochester, N.Y.) and counted in a scintillation counter (Beckman Instruments, Inc., Wakefield, Mass.). Average counts from lymphocytes in three control wells were deducted from average counts of lymphocytes in three wells with PHA.

Experimental design. For the initial passage, a 12-week-old SPF beagle was inoculated intravenously (i.v.) with 3000 TCID₉₀ of the attenuated CDV-RO. Five days later the dog was anaesthetized and a cervical lymph node was surgically removed. A portion of the lymph node was placed in MEM and ground in a Ten Broeck grinder to make a 10% (w/v) suspension. A second portion was stored frozen (−70 °C) for virus titration and assay in dog kidney cell (DKC) and macrophage cultures. One ml of the suspension was inoculated i.v. into a second dog (serial dog no. 2). Five days later a cervical lymph node was removed from the second dog. This procedure was continued for a total of eight serial passages.

Criteria for virulence of CDV included observation for clinical signs over a period of 21 days, daily rectal temperature recordings, weekly weight gains or losses, lymphocyte counts on p.i. days 0, 5, 7 and 14, blastogenesis responses of blood lymphocytes to PHA at weekly intervals, the presence or absence of virus antigen in epithelial cells of conjunctival imprints 8 days p.i. (Appel, 1969), the time of appearance of virus neutralizing antibody and amounts of virus from lymph node biopsies in DK as well as in DLM cell cultures as revealed by simultaneous titrations.

Two additional dogs were each inoculated i.v. with 1 ml of tissue culture containing CDV-RO that had been adapted and passaged ten times in DLM cultures as described. The inoculum had a virus titre of 10²⁵/ml when titrated in DLM cultures and of 10³⁵/ml when titrated in DK cells. Criteria used for judging the virulence of this virus were the same as described above.

RESULTS

Clinical signs

Clinical signs of distemper were not observed in dogs that received attenuated CDV in five serial in vivo transfers, however, animals inoculated with dog passages 6 and 7 had anorexia and marked depression by the second week p.i. This period was extended to the third week p.i. in the dog that received passage 8 virus. These animals also had conjunctivitis with watery discharge and diarrhoea that ranged in duration from 8 to 14 days p.i. The central or peripheral nervous system did not appear clinically affected. Only slight anorexia was observed in dogs inoculated with CDV after ten in vitro passages in DLM cultures. All dogs recovered clinically by the third week p.i.
Table 1. Weight gain of dogs after sequential inoculation of attenuated CDV

<table>
<thead>
<tr>
<th>Serial dog number</th>
<th>Post-infection (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>+0.8*</td>
<td>+0.6</td>
<td>+0.7</td>
<td>-0.2</td>
<td>-0.1</td>
<td>-0.9</td>
<td>-1.3</td>
<td>-1.9</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>+1.1</td>
<td>+1.0</td>
<td>+1.4</td>
<td>+0.6</td>
<td>-0.5</td>
<td>-1.0</td>
<td>-2.7</td>
<td>-2.6</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>+1.5</td>
<td>+1.6</td>
<td>+2.1</td>
<td>+0.8</td>
<td>+0</td>
<td>-0.4</td>
<td>-2.1</td>
<td>-3.8</td>
</tr>
</tbody>
</table>

* In kg. Weight gains or losses are based on the dog weight at the day of inoculation. Normal weight gains in 3-month-old beagle dogs are approx. 0.5 kg/week.

Table 2. Elevated rectal temperature response of dogs after sequential inoculation of attenuated CDV

<table>
<thead>
<tr>
<th>Serial dog number</th>
<th>Post-infection (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.8</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.6</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.6</td>
<td>39.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.8</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* In degrees Celsius. Only temperatures above 39.5 are listed (elevated body temperature in dogs).

Fig. 1. Peripheral blood lymphocyte counts from dogs after sequential inoculation of attenuated virus.
**Weight gains**

Weight gains were normal in those dogs that received lymph node suspensions from dog passages 1 to 3. However, weight losses were observed in later *in vivo* passages. They were most pronounced in the dogs given passages 7 and 8 (Table 1). Weight losses were also observed in dogs inoculated with DLM-passaged attenuated CDV.

**Elevated body temperature**

The temperature response pattern changed between dog passages 2 and 7 when elevated temperatures were observed. Responses became diphasic, typical of dogs inoculated with virulent CDV (Table 2). Elevated body temperature was also observed in dogs after inoculation with DLM-passaged CDV; it was diphasic in 1 dog.

**Lymphocyte counts**

Lymphocytopenia was observed in dogs beginning with dog passage 1 (Fig. 1). However, in dogs given material from the first three passages, reduced blood lymphocyte counts were restricted to day 5 p.i. They were equal or above the pre-inoculation values on days 7 and 14 p.i. Dogs inoculated with lymph node suspension from dogs representing passages 4 to 6 had reduced lymphocyte counts on days 5 and 7 p.i., but not on day 14 p.i. Lymphocytopenia extended into day 14 p.i. in dogs that received tissue passages 7 and 8 (Fig. 1). Lymphocyte counts in DLM-passaged CDV infected dogs were reduced on days 5 and 7 p.i. and approx. corresponded with *in vivo* passages 4 to 6.

**Lymphocyte blastogenesis**

A significant reduction in PHA lymphocyte stimulation was not seen until dog passage 5. Blastogenesis was reduced in blood lymphocytes 7 days p.i. in passages 5 to 8 and, in addition, in lymphocytes 14 days p.i. in passages 6 to 8. Recovery of mitogenesis was found in lymphocytes from these dogs by 21 days p.i. (Table 3). Stimulation was greatly reduced in dogs 7 days, but not 14 days p.i., with DLM-passaged CDV: 12.2 ± 3.2 and 16.5 ± 3.8 × 10^3 ct/min on day 0 p.i., 0.6 ± 0.2 and 0.4 ± 0.2 × 10^3 ct/min on day 7 p.i., and 16.6 ± 3.7 and 18.1 ± 2.4 × 10^3 ct/min on day 14 p.i.

**Virus antigen**

Virus antigen was not observed by immunofluorescence examinations 8 days p.i. in conjunctival imprints from dogs that were given virus passages 1 to 5. But, beginning with dog passage 6, virus antigen was seen in the cytoplasm of conjunctival epithelial cells. It was also found in imprints from both dogs inoculated with DLM-passaged CDV.

**Virus neutralizing antibody**

Antibody responses occurred at the anticipated time p.i. and at expected levels in all inoculated dogs, regardless of the passage (Table 4). Dogs inoculated with DLM-passaged CDV had comparable antibody responses.

**Virus replication in vitro**

During sequential CDV passages in dogs, an apparent change in the character of the original virus population occurred. CD virus titres from lymph node biopsies as measured in DK cells increased markedly in the first four passages but they decreased thereafter. Virus from passages 7 and 8 was not measurable in the DKC system. In contrast, titres obtained by assay in DLM cultures increased progressively after dog passage 3 (Table 5). Virus titres of less than 10^8 in early passages could not be measured because lymph node
Table 3. *Stimulation of blood lymphocytes with phytohaemagglutinin (PHA) in dogs after sequential inoculation of attenuated CDV*

<table>
<thead>
<tr>
<th>Serial dog number</th>
<th>Post-infection (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-7</td>
<td>13.7±2.9</td>
<td>17.1±2.0</td>
<td>11.2±1.6</td>
<td>16.0±2.1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>28.6±2.8</td>
<td>12.9±2.7</td>
<td>17.0±3.2</td>
<td>13.7±1.8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>16.2±2.3</td>
<td>9.2±2.3</td>
<td>6.9±0.9</td>
<td>10.6±1.3</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>14.8±3.1</td>
<td>8.9±1.8</td>
<td>12.8±1.5</td>
<td>12.2±2.1</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>10.6±1.5</td>
<td>10.0±1.7</td>
<td>13.1±1.7</td>
<td>14.7±1.5</td>
</tr>
</tbody>
</table>

* Average of three lymphocyte cultures with PHA minus average of three control cultures without PHA in ct/min × 10^-3.

Table 4. *Virus neutralizing antibody titres in dog serum after sequential inoculation of attenuated CDV*

<table>
<thead>
<tr>
<th>Serial dog number</th>
<th>Post-infection (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-7</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.0*</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.3</td>
<td>1.3</td>
<td>.</td>
<td>0.8</td>
<td>1.7</td>
<td>.</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.0</td>
<td>2.0</td>
<td>1.7</td>
<td>1.5</td>
<td>1.7</td>
<td>1.7</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.0</td>
<td>2.0</td>
<td>2.4</td>
<td>2.0</td>
<td>2.0</td>
<td>1.7</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>2.0</td>
<td>2.0</td>
<td>1.7</td>
<td>2.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* In log10 units.

Table 5. *Virus titration of CDV in lymph node biopsies* of dogs after sequential inoculation of attenuated CDV

<table>
<thead>
<tr>
<th>Serial dog number</th>
<th>Post-infection (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Virus titre in DLM† cultures</td>
<td>≤ 2.0†‡</td>
<td>≤ 2.0</td>
<td>≤ 2.0</td>
<td>2.5</td>
<td>3.0</td>
<td>2.9</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus titre in DK§ cell cultures</td>
<td>1.5</td>
<td>3.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.0</td>
<td>2.5</td>
<td>≤ 1.0</td>
</tr>
</tbody>
</table>

* Lymph node biopsies were taken from dogs 5 days after intravenous inoculation of lymph node suspension from the previous dog.
† Dog lung macrophage cultures.
‡ Virus titres in log10 of TCID/50 per 0.1 ml of inoculum.
§ Dog kidney cell cultures.
suspensions in dilutions less than 1:100 were toxic to macrophages and could not be tested for presence of virus in DLM cultures. A steady increase in virus titre in DLM cultures was observed between dog passages 4 and 8. Lymph node biopsies from dogs inoculated with DLM-passaged CDV had virus titres of $10^{3.5}$ and $10^{4.0}$ in DK cells and $10^{2.9}$ and $10^{3.4}$ in DLM cultures.

A difference in titration endpoints in DLM cultures was not found when syncytial formation and IF results were compared.

DISCUSSION

Reversion to virulence of an attenuated CDV strain (Rockborn) was established in this study. After approx. six sequential passages in dogs as well as after ten passages in DLM cultures in vitro, the virus was no longer avirulent, by the criteria employed. Eight criteria for assessing virulence were used. These included clinical signs, weight gain or loss, elevated body temperature, blood lymphocyte numbers, mitogen blastogenesis responses of blood lymphocytes, appearance of CD virus antigen in surface epithelium, neutralizing antibody responses, and virus replication in DK cells versus DLM cultures. A shift to greater virulence was found in seven of the eight criteria. A change was not seen in time of appearance and titres of neutralizing antibody. It should be noted that all dogs survived; but virulent CDV given by aerosol or i.v. routes of inoculation induced only a 50% mortality in dogs (Appel, 1969). Earlier studies with CDV in dogs established a relationship between the early presence of neutralizing antibody and survival in dogs given experimental inoculations of virulent virus by the aerosol or i.v. route (Appel, 1969). With continued virus passages in dogs, delay or lack of antibody formation with a fatal outcome in some dogs may have been expected in this study.

Reversion to virulence of attenuated virus in infected animals has been observed frequently (reviewed by Clark, 1978). Only one report has been made in regard to CDV. Clinical signs and mortality have been used as criteria for reversion to virulence in a study made in ferrets. Goto et al. (1976) reported reversion to virulence of an egg adapted CDV strain in ferrets. Spleen and lung suspensions were transmitted at 6- to 7-day intervals. Clinical signs in ferrets were seen after 14 passages; after 22 passages all ferrets died. Mortality rates after CDV infection are much higher in ferrets than in dogs (Appel & Gillespie, 1972).

The difference in virulence of CDV in this study could not be related to the amount of virus present in lymphatic tissue. Virus titres in lymph node tissues from dogs in passages 2 to 4 were as high or higher, by titration in DK cells, than lymph node virus titres from dogs representing passages 5 to 7 that were made in macrophage cultures. It may be assumed that a virus population of increased virulence emerged during passages in dogs and in macrophage cultures. This population, which preferentially replicated in macrophage cultures in vitro, became dominant, while the attenuated virus population, which grew better in DK cells, diminished.

The correlation between virus replication in macrophages and virulence has been documented for several virus infections (Bang & Warwick, 1960; Mims, 1964; Stevens & Cook, 1971; Olson et al. 1975). Of particular interest in this connection may be an observation by Sullivan et al. (1975), who suggested that higher measles virus (MV) susceptibility of monocytes in neonates would increase virulence of MV. Measles virus is closely related to CDV (Imagawa et al. 1960).
The difference in infectivity of virus populations might be explained in several ways. Maturation of virus envelope glycoproteins could be important factors for infectivity, as has been shown with Sendai virus and Newcastle disease virus (NDV). Complete infectious Sendai virus was produced in embryonated eggs, but only after trypsin treatment in epithelial cell lines (Homma & Ohuchi, 1973). Proteolytic cleavage of precursor glycoproteins F0 and HNo into mature glycoproteins F and HN occurred more readily in virulent than in avirulent NDV (Nagai et al. 1976). Similar effects may be found in CDV infected cells and should be investigated. However, virulent CDV matured in macrophages does not readily infect epithelial cells in vitro, which would be difficult to explain on that basis.

Incorporation of cellular compounds into virus by budding through membranes of a particular cell might increase infectivity for the same cell type. Replication of CDV in macrophages might be required for a virulent virus population for that reason. However, virulent CDV derived from epithelial cells of infected dogs, as, for example, from surface scrapings of urinary bladder or conjunctiva, can readily infect and replicate in macrophages in vitro (M. Appel, unpublished data). It appears, therefore, that maturation of CDV in a particular cell membrane is less important for selection of virulence than selective replication of virulent virus clones.

Similar conclusions were made in other studies where an increase in virulence of attenuated virus populations was observed in serial passages in vitro. Clark (1978) found that attenuated rabies became virulent for mice after several passages in neuroblastoma cell cultures. Attenuated enterovirus, influenza, and togavirus virulence was increased in cell cultures incubated either at supraphysiological temperatures or, if attenuated virus was not serially cloned, after isolation (reviewed by Clark, 1978).

The author gratefully acknowledges the assistance of Mary Beth Metzgar and Ann Signore. This work was supported by the Whitehall Foundation.

REFERENCES

Reversion to virulence of attenuated virus


(Received 14 April 1978)