Virulence of Tissue Culture-Propagated Canine Distemper Virus

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Virulence of canine distemper virus (CDV) adapted to in vitro growth in Vero or bovine cells was determined by inoculation into CDV-susceptible neonatal gnotobiotic dogs. When compared with dogs given virulent R252-CDV, Vero R252-CDV was attenuated at passage level 14. In contrast, dogs inoculated with bovine R252-CDV at the same passage level experienced rapid fatal neurological disease. Virulence was not linked to ability to infect or replicate in canine pulmonary macrophage cultures. Retention of virulence by bovine R252-CDV is unique and worthy of further study.

Tissue culture-propagated viruses vary greatly in their virulence for experimental or natural hosts. For some agents such as scrapie, virulence is not greatly affected by passage in culture (11). Other agents, however, are rapidly attenuated by in vitro passage, and this feature has permitted the development and use of modified live viral vaccine products.

Although virulence (capacity to induce disease) is affected by a great many host factors, such as age, immunocompetence and immunity, and nutrition and pregnancy, markers for viral determinants of virulence are less well defined. For some viruses, physicochemical properties of virion proteins are determinants of pathogenicity. Recent work with various subtypes of avian influenza viruses has indicated that the structure of the hemagglutinin, including its susceptibility to proteolytic cleavage, is the determining factor for virulence (26). Another in vitro factor influencing virulence is the type of cell system used to propagate the virus (9, 14). Two general types of cells exist for viruses: permissive and nonpermissive. Permissive cells readily support viral growth without a period of adaptation, and virulence is more likely to be retained in these cells. Nonpermissive cell systems do not support initial viral replication in vitro but readily support viral infection after the inoculum has been adapted to these cells via repeated blind passages.

Virulent canine distemper virus (CDV), a parvovirus pathogen of dogs, is readily propagated in primary canine pulmonary macrophage (PM) cultures (3, 22), a permissive cell system. Virulence has been retained throughout 38 in vitro passages on these cells (3). Viral propagation in epithelium-type cultures such as homologous canine kidney or heterologous African green monkey kidney (Vero) cells is accomplished with difficulty during initial isolation but is easily performed after adaptation (2, 10, 23). Once adapted to these nonpermissive cells, CDV attenuates rapidly, losing both virulence for dogs (1, 5, 6, 24) and capacity to infect PM (1). This concept of permissive versus nonpermissive cell systems has received support from studies by Appel, who demonstrated that attenuated (Rockborn strain) CDV reverts to virulence upon repeated passage of the virus through CDV-susceptible dogs (1). In that study, reversion to virulence coincided with the required capacity of the virus to replicate in PM cultures.

For the past several years, this laboratory has investigated mechanisms of neurological disease caused by CDV infection. Most of these studies have used a viral biotype (R252-CDV) isolated from a dog with chronic demyelinating encephalomyelitis (20). The virus has been adapted to growth in Vero cells (10). However, unlike attenuated laboratory strains (Rockborn and Ondersteboort), Vero-adapted R252-CDV (V-R252-CDV) readily replicates to high titers on PM cultures (A. E. Metzler, unpublished data). Recently, R252-CDV has been adapted to growth in primary cultures of bovine cells obtained from jugular venipuncture (A. E. Metzler et al., Arch. Virol., in press). Further, bovine cell-adapted R252-CDV (B-R252-CDV), like V-R252-CDV, also grows readily in canine PM cultures. Although both isolates replicate in PM cultures, in vivo virulence for dogs has not been determined. The objective of this study was to determine whether V- and B-R252-CDVs retain virulence for dogs and to compare various pa-

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rameters of infection in gnotobiotic dogs given nontissue culture- and tissue culture-propagated R252-CDV.

MATERIALS AND METHODS

Dogs, viruses, and routes of inoculation. A total of 17 gnotobiotic dogs (18, 19) from three litters were used in this study. All dogs were inoculated with various viral inocula at 6 to 9 days of age. Six dogs (group 1) received 0.2 ml of virulent R252-CDV (10% spleen-thymus homogenate) intraperitoneally. The inoculum contained 10<sup>2.5</sup>-10<sup>5</sup> 50% PM tissue culture infective doses per ml. Four dogs (group 2) received 0.2 ml of 14th in vitro passaged V-R252-CDV intracerebrally (n = 3) or intraperitoneally (n = 1). The infectious titers were 10<sup>4.5</sup>-50% tissue culture infective doses per ml on Vero cells and 10<sup>4.5</sup>-50% tissue culture infective doses per ml on PM. Five dogs in group 3 received passage 14 B-R252-CDV (2.5 x 10<sup>5</sup> to 10 x 10<sup>6</sup> virus-infected whole cells) intracerebrally. The titer of this inoculum (passage 15) on FM cultures was 10<sup>2.5</sup>-50% PM tissue culture infective doses per 10 x 10<sup>4</sup> cells. Infectivity was not found when B-R252-CDV was titrated on Vero cells. In addition, two litters were given uninfected bovine cells (8 x 10<sup>4</sup> and 32 x 10<sup>4</sup> uninfected whole cells) as controls.

Parameters of virulence in vivo. Unclotted blood samples were collected weekly for determination of absolute lymphocyte levels. Viremia was determined by the direct immunofluorescence method on acetone-fixed smears of leukocytes (15). In addition, heparinized blood samples were collected and leukocytes were isolated by dextran (6%, wt/vol) sedimentation. In vitro responses of these cells to the plant lectins phytohemagglutinin-P and pokeweed mitogen were determined as described in previous publications (15, 21).

Virus-neutralizing antibody titers in serum samples were determined in a microtiter neutralization assay with Onderstepoort strain CDV essentially as described by Appel and Robson (4).

Parameters of virulence at necropsy. At the termination of the experiment or when moribund, the dogs were sacrificed. Tissues were examined for evidence of viral infection by virus-specific immunofluorescence on nervous tissues (18) or by histopathology, or by both.

For virus resolation from brain, explant cultures from the cerebellum, temporal lobe, and left cerebral hemisphere were established in 25-cm<sup>2</sup> disposable plastic flasks (Corning Glass Works, Corning, N.Y.) containing Eagle minimal essential medium with 20% fetal bovine serum. Explants were subcultured on glass slides (no. 4804, Lab Tek Products, Miles Laboratories, Naperville, Ill.) and examined for viral antigen by immunofluorescence and for virus-specific cytopathic effects on hematoxylin and eosin-stained monolayers.

RESULTS

Parameters of virulence in vivo. With two exceptions (Table 1), serum samples obtained from puppies before inoculation with various CDV variants were free of virus-neutralizing antibodies. Results summarizing the effect of CDV of different passage histories on various parameters of infection are given in Table 1. Five dogs received V-R252-CDV. A transient lymphopenia was observed on post-infection day (PID) 7 only. This was not associated with viremia as measured by immunofluorescence. These dogs did not become ill during the period of observation, and serum contained virus-neutralizing antibodies at levels associated with recovery (mean = 1:203) by PID 28. One dog, however, died of a congenital heart defect unrelated to viral inoculation on PID 7.

In contrast, five dogs given B-R252-CDV at the same in vitro passage level experienced progressive and fatal CDV infection. This was manifested by progressive lymphopenia and viremia and lack of virus-neutralizing antibodies in terminal serum samples. One dog died of acute neurological dysfunction on PID 10, and the

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Virulent R252-CDV</th>
<th>B-R252-CDV</th>
<th>V-R252-CDV</th>
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<tr>
<td></td>
<td>Lymphocytes (per mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>Viremia (IF&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>VN antibody&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>5,787 ± 1,245&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;1:2</td>
<td>3,423 ± 77</td>
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<tr>
<td>6-7</td>
<td>1,181 ± 519</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1,582 ± 238</td>
</tr>
<tr>
<td>12-14</td>
<td>471 ± 124</td>
<td>&lt;1:2</td>
<td>700 ± 48</td>
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<tr>
<td>21</td>
<td>195 ± 58</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Dead</td>
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<tr>
<td>27-28</td>
<td>234 ± 106</td>
<td>± &lt;1:2</td>
<td>Dead</td>
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<sup>a</sup> IF, Immunofluorescence.
<sup>b</sup> VN, Virus neutralizing.
<sup>c</sup> Mean ± standard error of the mean.
<sup>d</sup> Two of five dogs tested had virus-neutralizing antibody of 1:2 and 1:3; the remaining dogs did not contain antibody at 1:4 (lowest dilution tested).
<sup>NT</sup> Not tested.
remaining four dogs died on PIDs 14 to 18. Dogs receiving uninfected control bovine cells did not exhibit clinical signs of disease, and no changes in parameters of infection were observed.

As documented elsewhere (16), dogs receiving virulent R252-CDV became lymphopenic on PID 7 and this response, associated with progressive viremia and failure to produce virus-neutralizing antibodies, persisted throughout the experimental period until death from CDV-related encephalitis (PIDs 27 to 32).

In general, the effects of various viral inocula on in vitro phytomitogen responses of isolated leukocytes paralleled hematological data (Fig. 1). Lymphocytes from dogs receiving virulent R252-CDV were unresponsive to either phytohemagglutinin-P or pokeweed mitogen on PID 7 and remained unresponsive until death. Similarly, lymphocytes from B-R252-CDV-infected dogs did not respond to phytohemagglutinin-P, although a modest response to pokeweed mitogen was retained. Lymphocytes from dogs given V-R252-CDV experienced a mild and transient suppression of phytomitogen responses after infection. Suppression, however, was not complete and had recovered to the normal range by PID 21.

Parameters of virulence at necropsy. Dogs receiving virulent R252-CDV had lesions typical of fatal CDV infection, i.e., systemic lymphoid depletion and interstitial pneumonia (2). In the brains, viral antigen (immunofluorescence) was demonstrated in the frontal lobe, periventricular region of the fourth ventricle, and cerebellum. Microscopic lesions in the brains of these animals consisted of small foci of neuronal degeneration and accompanying microgliosis. CDV was readily reisolated from explant cultures and identified as such by immunofluorescence and viral cytopathic effect.

Dogs receiving B-R252-CDV had lesions in the brain and elsewhere similar to those given virulent R252-CDV. Explant cultures prepared from dogs dying on PIDs 10 and 14 displayed virus-specific changes (i.e., immunofluorescence and cytopathic effect). Explants prepared from the controls receiving uninfected bovine cells were virus negative.

In contrast, dogs receiving V-R252-CDV did not have CDV-associated lesions at necropsy. Thymic and peripheral lymphoid tissues were intact and comparable in size and morphological features to those of uninfected gnotobiotic dogs of that age. Neither lesions nor viral antigens were observed in the brains of these animals. Virus was not recovered from these animals with the explant technique described above.

**DISCUSSION**

The present study was designed to determine whether R252-CDV adapted to in vitro growth in cells of monkey (Vero) or bovine origin retains virulence for CDV-susceptible gnotobiotic puppies and to test the hypothesis developed by Appel in which capacity to cause disease was linked to efficient in vitro growth in canine PM.

![FIG. 1. Effects of infection with virulent CDV and V- and B-R252-CDV upon tritiated thymidine incorporation by lymphocytes cultured in vitro with phytomitogens.](http://iai.asm.org/)
cultures (1). In that study, the Rockborn strain of CDV grew poorly on PM initially, but serial passage in dogs or in PM resulted in a reversion to virulence. Reversion was directly correlated with ability to replicate in PM. In our studies, both V- and B-R252-CDVs readily infected canine PM in vitro. Titration experiments with these viruses on PM revealed infectious titers comparable to that of virulent R252-CDV on PM monolayers. Moreover, B-R252-CDV did not display infectivity when assayed on Vero cells.

The data presented indicate that V-R252-CDV was attenuated during passage in tissue culture, whereas B-R252-CDV was not. In fact, the rapid onset of fatal disease (PIDs 10 to 18) in dogs given B-R252-CDV suggests that compared with dogs given virulent R252-CDV, in vitro passage in these cells enhanced neurovireulence. One could argue that the nature of the inoculum (virus-infected whole cells) might have influenced the outcome of infection as reported for the defective measles virus of subacute sclerosing panencephalitis (7). However, this is unlikely since infected bovine cells released infectious progeny virus, although with a restricted in vitro host cell range. Thus, our virus is not defective as are subacute sclerosing panencephalitis viral isolates. This point is discussed in detail elsewhere (Metzler et al., Arch. Virol., in press).

A number of investigators have reported that adaption of CDV to growth in embryonic eggs (8), dog kidney (6, 24), or BSC-1 (12) cultures changes virulence for dogs or ferrets. These studies have indicated that attenuation occurs by passage 20 or at an undefined lower level (5). Although mechanisms are undetermined, it is likely, considering data obtained from virulent and attenuated R252-CDV-infected bovine cells (Metzler et al., Arch. Virol., in press) and other virus-host systems, that the process of in vitro passage "selects" viral variants from the original inoculum for its capacity to grow in different cells. Loss of virulence is thus explained by the fact that virulence and in vitro growth are genetically disparate processes of the virion.

Vero cells are of epithelial (kidney) origin and are somewhat similar to dog kidney cells in their capacity to support CDV. The bovine cells were derived from a proliferative cell occurring in peripheral blood monocyte cultures. Although the origin of these cells is unclear, they do not phagocytose latex beads or express cell membrane receptors for Fc portions of immunoglobulin or the activated third component of complement, characteristics of macrophages (13). It is therefore likely, though not proven, that these cells represent progeny from cells contaminating the needle during the jugular venipuncture procedure (Metzler, unpublished data). Whatever their origin, the fact that cells of non-Canidae origin can support virulent virus growth in vitro is unique and worthy of further investigation.

When compared with effects of modified live virus vaccination in susceptible dogs, V-R252-CDV was not, however, completely innocuous. Dogs receiving modified live virus CDV do not become lymphopenic and their lymphocytes remain responsive to phytoagglutinins (1, 25). Thus, V-R252-CDV exhibits a graded effect upon these parameters and is intermediate between modified live virus CDV and fully virulent R252-CDV in its effects on lymphoid function. This phenomenon is probably a reflection of the ability of V-R252-CDV to infect canine PM in vitro. The overall significance of this observation must be minimal since it was demonstrated that involvement of nonlymphoid tissues (e.g., the brain) was not observed.

One area of investigation currently under study in this laboratory is a search for viral markers of virulence as reflected in virion polypeptide composition. If low-passage V-R252-CDV were virulent for dogs, large amounts of viral material could be obtained from this culture system, thereby enabling subsequent comparisons with attenuated laboratory strains of CDV such as Ondersteapoort. The results of this study indicate that V-R252-CDV (even at low passage level) is no longer virulent for neonatal CDV-susceptible gnotobiotic dogs. Therefore, comparative analyses (i.e., immunoprecipitation of metabolically radiolabeled viral proteins followed by polyacrylamide gel electrophoresis) have to be performed with either PM or bovine cell-propagated virulent virus.

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LITERATURE CITED