In vitro and in vivo Properties of the Virus Causing Natural Canine Distemper Encephalitis

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SUMMARY

A group of dogs with naturally occurring canine distemper developed prodromal systemic symptoms followed by neurological disorders. The post-infection courses of these diseases lasted approximately 2 months. A varying degree of demyelination and inclusion body formation was found mostly in the cerebella of virologically confirmed cases with little or no inflammatory response. The distribution of canine distemper virus antigen coincided with the histopathological lesions. The animals had moderate to high neutralizing titres to the virus in their sera and a low level of interferon-like activity in their cerebrospinal fluids. Isolation of viruses was most successful by the cocultivation method for brain specimens, but was possible by the direct method using lung homogenates. In infected Vero cells, the isolates derived from brain caused the formation of distinct plaques consisting of multinucleate giant cells, but the isolates from lung induced a cytopathic effect mainly consisting of cell rounding which eventually spread throughout the culture. The former infection produced less extracellular virus than the latter. The synthesis of the viral surface proteins H and F, and of M, was markedly reduced compared with that of the internal viral proteins such as NP, P and L. The SDS–PAGE migration pattern of the P protein varied from case to case, but was similar when isolates from different tissues of the same case were compared. In the affected tissues, the amount of viral polypeptides decreased markedly relative to that of the NP and there was also an absolute decrease compared to their abundance in Vero cells. This decrease was more obvious in the brain than in the lung. The relevance of these results is discussed.

INTRODUCTION

Measles virus (MV) and canine distemper virus (CDV), belonging to the genus Morbillivirus, are highly contagious pathogens causing acute systemic infections (Kempe & Fulginiti, 1965; Appel, 1969) and, rarely, chronic infections involving the central nervous system (CNS) (Yamanouchi, 1980). This variety of clinical manifestations appears to reflect different virus–cell interactions. The association of these viruses with slow infections such as subacute sclerosing panencephalitis (SSPE) (Connolly et al., 1967; Baublis & Payne, 1968) and old dog encephalitis (ODE) (Lincoln et al., 1971; Imagawa et al., 1980) has attracted increasing attention and led to the investigation of the virus–cell interactions by which these viruses can establish persistent infection with relative ease both in vitro and in vivo (Fraser & Martin, 1978; ter Meulen & Carter, 1984).

Many comparative studies using isolates from brain tissues of chronic neurological cases and their counterparts from non-neural tissues of acute uncomplicated cases have been performed mainly in various cell lines, and differences in their properties have been found through various virological approaches (Morgan & Rapp, 1977; Wechsler & Meissner, 1982; Shapshak et al., 1982; Tobler & Imagawa, 1984). Although the variation in properties ranges widely among isolates from SSPE patients, the most important general characteristic of the isolates which is relevant to persistent infection appears to be the viral propensity for cell association (Doi et al., 1972; Thormar et al., 1973; Burnstein et al., 1974). A major reason for the cell association
differences has been attributed to the isolates themselves, some of which were impaired in the production of certain viral polypeptides, and in particular the normal M protein (Wechsler & Fields, 1978; Hall & Choppin, 1979; Lin & Thormar, 1980; Young et al., 1985; Sheppard et al., 1985). If this is generally the case, much other biological and morphological evidence obtained from studies in vitro using the cell-associated SSPE viruses can be successfully explained. Conversely, replication of MV is controlled by certain cellular conditions (Robbins & Rapp, 1980; Miller & Carrigan, 1982; Joseph et al., 1975); host cell factors and specific combinations of virus and cell may play an important role in determining the mode of infection by morbilliviruses (Wild & Dugre, 1978; Wechsler & Meissner, 1982).

Therefore, studies are needed to examine whether the putative molecular mechanisms operating during persistent infection in vivo by morbilliviruses can be generalized to persistent infection in vitro by morbilliviruses, and to elucidate the relative significance of viral and host cell factors in establishing persistent infection in vivo. In spite of the similarity between clinical findings in cases of SSPE and ODE, their respective virus isolates have different properties in vitro, for instance polypeptide synthesis (Lin & Thormar, 1980; Hall et al., 1979) and the cell association property (Doi et al., 1972; Imagawa et al., 1980). These results, however, were obtained from only temporary infections in vitro. Since the co-cultivation method exclusively provides excellent results in CDV isolation from the CNS of dogs with chronic distemper encephalitis, the study of these cases may facilitate the study of the problems pointed out above. It is of particular importance to compare the properties of CDV in the critical phase when persistent infection is just being established in vivo with infection in vitro.

In the present paper, I describe the results obtained from the following investigations of transitional cases of canine distemper encephalitis: (i) clinical and histopathological examinations, (ii) virus neutralization by sera, cerebrospinal fluids (CSF) and tissue homogenates, (iii) interferon (IFN) assays of CSF, (iv) c.p.e. and growth curves of the isolates, (v) analysis of viral polypeptides both in vitro in Vero cells infected with the isolates and in vivo in the affected tissues.

**METHODS**

**Dogs and collection of specimens.** Twelve dogs with spontaneously occurring neurological signs were used in this study. Their clinical courses are outlined in Fig. 1. At autopsy, various specimens including CNS (pallium, brain stem, cerebellum), lung, blood and CSF were aseptically obtained immediately after euthanasia. A hemisphere and a part of the lung were immersed in 10% formal saline for histopathological examination. Other parts of the tissues, the blood and the CSF were cooled immediately on ice and processed for virus isolation and immunofluorescence studies within 2 h after their collection. The samples were stored at -70°C until use.

**Cells and viruses.** Vero cells were grown in a mixture of equal volumes of Eagle's MEM and YLE (Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate and 0.1% yeast extract) supplemented with 5% foetal calf serum. Primary cell cultures (pDK) were prepared from baby dog kidneys by a standard method and grown in the same medium. The DFE strain of CDV (Ochi et al., 1960) was kindly supplied by the Biochemical Co. (Kyoto, Japan). This reference strain was originally isolated after subcutaneous injection of a ferret with a mixture of lung and spleen homogenates collected from a dog which had died from neurological disease 7 days after onset of the disease. The isolate was passed three further times in ferrets using affected spleen homogenates intraperitoneally, 27 times in chicken choioallantoic membrane, and three times in Vero cells.

A large number of CDV isolates were obtained from the dogs in the present study. The isolates were maintained by co-cultivation of infected and uninfected cells during early passages and were stored by freezing infected cells in 10% dimethyl sulphoxide at -70°C. The New Jersey strain of vesicular stomatitis virus (VSV) was supplied by the National Institute of Animal Health of Japan (Tokyo, Japan) and was passaged twice in pDK cells.

**Antisera.** A ferret was infected by a single inhalation of a suspension of the DFE strain and bled 4 weeks later. The antiserum obtained had a CDV neutralizing antibody titre (NT) of 1:2000. Antiserum to ferret IgG was prepared in a rabbit by injecting it three times at 3 week intervals into the legs intramuscularly and several sites on the back subcutaneously with emulsions of purified ferret IgG and Freund's incomplete adjuvant (Difco). Serum was taken 2 weeks after the second booster and had a highly specific precipitin antibody titre of 1:32. These antisera were used for detection of viral polypeptides after absorption with acetone-extracted powders of dog brain tissues and Vero cells. Ferret IgG for use as an immunogen was purified by precipitation with 33% ammonium sulphate, gel filtration through Sephacryl S-300 (Pharmacia) and ion-exchange chromatography on DEAE-Sephabel (Pharmacia) at a salt concentration of 17 mM. Another antiserum to CDV was prepared by immunizing a rabbit with virus purified by potassium tartrate gradient centrifugation. Anti-rabbit IgG antiserum conjugated
with fluorescein isothiocyanate (FITC) was purchased from Miles Laboratories. These antisera were used for immunofluorescence staining after the absorption.

Preparation of samples for SDS–PAGE. Cultures infected with the isolates were prepared by co-cultivating infected and uninfected Vero cells in 3 cm Petri dishes. Vero cells were infected with DFE strain at a m.o.i. of 1. When more than 80% of these cultured cells showed c.p.e. 2 to 3 days post-infection, they were radiolabelled in Hanks' salt solution containing a U-14C-L-amino acid mixture (NEC-445, New England Nuclear) at 5 μCi/0.5 ml/3 cm dish for 30 min after preliminary starvation. The labelled cells were rinsed and precipitated with trichloroacetic acid (TCA) and acetone. The dried cells were lysed in 50 μl of gel sample buffer (Laemmli, 1970) and the lysates were boiled for 3 min. For radioimmunodetection, the infected cultures were prepared simultaneously with and processed identically to the labelled cultures, except that the isotopic labelling was omitted. Tissue blocks (5 mm × 7 mm) were sliced into sections 15 μm thick. Fifty sections per tissue were suspended in 500 μl of phosphate-buffered saline and were disrupted with a vortex mixer and a sonicator for 30 s each. The suspensions were processed in the same way as the cultured cells and lysed in 100 μl of the gel sample buffer. The lysates were stored at −20 °C until use.

Histopathological and immunofluorescence examinations. Tissue sections were cut 5 μm thick. Lung sections were stained with haematoxylin and eosin (HE), and consecutive brain sections were stained with HE and luxol fast blue. Frozen sections (10 μm) cut in a cryostat (SLEE Ltd, U.K.) and coverslip cultures were treated with cold acetone for 15 min, and then overlaid with drops of the anti-CDV rabbit antiserum at 37 °C overnight followed by the FITC-conjugated anti-rabbit IgG antiserum at 37 °C for 1 h. The excess primary and secondary antisera were removed by washing three times. The cells were examined under a fluorescence microscope (Olympus).

Virus isolation. Three different methods were used for virus isolation from brain tissues. Five ml of brain cell suspension was prepared by trypsinizing minced fragments of a tissue block (0.5 g) and each half of the suspension was inoculated into a 2 oz. culture bottle containing either 10 ml of growth medium only or subconfluent Vero cells. Alternatively, the tissue (0.5 g) was homogenized in a mortar with 5 ml of Hanks' salt solution. After centrifugation of one half of the homogenate, the supernatant was inoculated into Vero cell culture. The culture was allowed to adsorb virus at 37 °C for 1 h and was fed with 10 ml of growth medium after removal of the inoculum. 

Heparinized blood, the CSF and the lung tissue homogenate were tested by the same method as described for the brain tissue homogenate. The inoculated cultures were incubated at 35 °C and examined daily under a microscope for evidence of viral c.p.e. for 20 days, during which time they were refreshed by medium exchange every 5 days and subcultured on the 10th day of the incubation. The residual samples were stored at −70 °C.

Infectivity assay and virus neutralization. Virus infectivity was assayed using serial tenfold dilutions of virus suspension and Vero cells grown in microtitre wells. A 0.2 ml amount of each dilution was inoculated into each of four culture wells. The neutralizing antibody titre was determined by a micromethod instead of the tube culture method (Shishido et al., 1967). Both titres were calculated on the basis of the results obtained on the 10th day of incubation.

Growth curves of extracellular virus. Extracellular virus growth was compared between the DFE strain and isolates from the brain and the lung of each case. Vero cells grown in 2 oz. culture bottles were infected with either of these viruses at a m.o.i. of 0.01. After adsorption followed by three washes to remove the inoculum, the cultures were fed with 10 ml of growth medium and incubated at 35 °C. One half of the medium was replaced by fresh medium at designated times (0, 2, 4, 6 and 8 days post-infection). The collected samples of medium were stored at −70 °C after centrifugation and the supernatants were tested for infectivity all at the same time.

Interferon assay. Interferon was assayed according to the method of Tsai et al. (1982) modified for use with pDK cells and approximately 25 p.f.u. of VSV.

Radioiodination. The rabbit IgG fraction was purified from the anti-ferret IgG antiserum by the same methods as described above. Ten mg of this purified IgG was radiolabelled with 2 mCi Na125I (NEZ-033A, New England Nuclear) by the chloramine-T method (Graye et al., 1977).

SDS–PAGE. This was performed according to the method of Laemmli (1970), using 10% polyacrylamide slab gels (10 cm × 1.5 mm) at a constant voltage of 70 V for 15 h. Gels containing samples of the radiolabelled cultures were processed for autoradiography and exposed for 7 days. Gels containing non-radioactive specimens were subjected immediately to Western blotting; the viral polypeptides transferred to a nitrocellulose membrane (NC, Bio-Rad) were detected by autoradiography of the NC after being processed for radioimmunological reactions. Molecular weights were estimated by comparison with reference proteins of known molecular weight (Boehringer).

Western blotting and radioimmunodetection. Electrophoretic Western blotting was performed using a Trans-Blot Cell and a Model 160/1.6 power supply (Bio-Rad). The gel and the NC were put together tightly and then were immersed in 20 mM-Tris/150 mM-glycine/20% methanol. Proteins were transferred from the gel to the NC by vertical electrophoresis at a constant 70 V for 6 h. Unoccupied sites on the NC were saturated with 5% bovine serum albumin. The NC was immersed in the anti-CDV ferret antiserum (1:10), allowed to react with continuous rocking at room temperature for 1 h followed by rocking at 4 °C overnight, and washed five times. The second cycle of the reaction was done with the 125I-labelled anti-ferret IgG antiserum (106 c.p.m./10 ml per membrane) for 4 h. The NC was processed for autoradiography.

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RESULTS

In order to study the mechanism of virus persistence in dogs with canine distemper encephalitis, it was essential to confirm first of all that their neurological diseases were caused by CDV and to know the post-infection course of the diseases.

Clinical observations

The dogs investigated in the present study were diagnosed clinically as cases of canine distemper and were under treatment at the Department of Veterinary Internal Medicine, Faculty of Agriculture, Osaka Prefectural University. When they became seriously ill or moribund, euthanasia was performed at their owners' request because of the prognosis of a fatal outcome or severe sequelae. Symptoms and their sequential appearance during the clinical course of the disease are outlined in Fig. 1. The ages of the dogs varied up to 6 years old. Eleven of 12 cases had not received a distemper vaccine and the vaccine status of Case 1 was not certain. Since clinical records were not available earlier than the first clinical examination (day 0), the symptoms are described from their owners' accounts. In general, most of the cases developed the prodromal symptoms of canine distemper such as fever, cough, vomiting, diarrhoea and anorexia several weeks before day 0. They had already suffered the hyperkeratosis (hard pad) pathognomonic of canine distemper before day 0. As the disease progressed, the neurological symptoms of convulsion, motor inco-ordination and ataxia successively appeared. The period from natural infection to euthanasia was estimated to be approximately 2 months or more in most instances except Cases 1 and 9 for which no information was available before day 0. Thus, the infection appeared to lie in the transitional phase between acute and persistent when the animals were autopsied.
### Table 1. Results of laboratory examinations

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* Cases 6 and 14 were healthy dogs.
† NT, Not tested.
‡ Undetectable level in undiluted CSF.
Laboratory examinations

Sera taken from the cases at the time of autopsy were tested for neutralizing antibody to CDV (Table 1). For the confirmed cases, NT titres varied from intermediate (32 to 128) to high (326 to 1024). Cases 1 to 3, 5, 8 and 16 developed intermediate antibody responses.

Interferon-like activity was assayed in CSF collected at the time of autopsy when the infection had already passed the critical phase (20 days post-infection; Krakowka et al., 1975) (Table 1). A low level of IFN-like activity was detected in the CSF of only those dogs from whose brains infectious CDV was recovered, with the exception of Case 5, but it was not demonstrated in the CSF of dogs negative for virus isolation or in that of healthy dogs.

Three parts of the brain and the lung were examined histopathologically and tested for CDV antigen by indirect immunofluorescent staining. In general, histopathological changes mainly consisted of demyelination and inclusion body formation with little inflammatory response as judged by cellular infiltration, and were frequently most severe in the cerebellum, followed by the brain stem and the pallium (Table 1, Fig. 2). The topographical distribution and the relative intensity of immunofluorescence correlated well with those of the histopathological changes (Table 1). Intense immunofluorescence was predominantly observed in the cytoplasm of glial cells of the cerebellar white matter (Fig. 3) and the epithelial cells of the lung.

Virus isolation

Because of the supposed cell association of the causative virus, virus isolation was performed using various kinds of tissue specimens and methods including cultivation of the affected brain cells with (co-cultivation method) and without Vero cells (plain cultivation method) in addition to the standard method where tissue homogenate was inoculated onto subconfluent Vero cells. Table 2 summarizes the results of virus isolation. A highly successful rate of virus isolation from the brains (9/12) of affected dogs was accomplished by the co-cultivation method. The three different parts of a single brain gave the same result, apart from two specimens with bacterial contamination. The isolates induced morphologically different types of viral c.p.e. depending on the tissue from which they had been derived. The morphology of the c.p.e. will be described in detail below. In the plain cultivation method it was difficult to make trypsinized brain cells grow and form a confluent monolayer in which multinucleate giant cells (MGC) could be formed by the fusion activity of the causative virus. For this reason, about half of the cases remained undetermined as to virus isolation by this method. As far as the plain cultivation succeeded, it gave positive results for five of six cases, which is comparable to the rate achieved by the co-cultivation method although there were some discrepancies in the results for different parts of

Fig 2. Histopathological changes observed in a cerebellar lesion of Case 3. (a) Marked demyelinating lesion. (b) Inclusion body formation (arrows).
Properties of encephalitogenic CDV in vivo

Table 2. Results of virus isolation

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<td>+</td>
</tr>
<tr>
<td>Blood</td>
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</tr>
</tbody>
</table>

* Cases 6 and 14 were healthy dogs.
† BC, Bacterial contamination.
$^\dagger$ NG, No cell growth.
§ NT, Not tested.

the same brain. MGC were observed in plain cultures of affected brain tissues (Fig. 4). In contrast, by the ordinary method virus was not detected in almost all homogenates of the brains even though these had been found positive by the co-cultivation method. The exceptions were two cerebellar homogenates of Cases 3 and 16 which gave positive results. For testing the lung samples, only tissue homogenates were used and tested by the ordinary method. Six out of eight lungs of the confirmed cases gave positive results. Cases 4 and 12 each showed differences between their brain and lung samples, which had high NT titres in their sera. Virus was also efficiently isolated from CSF (7/12) in complete agreement with the results of virus isolation from the lung homogenates. No virus was recovered from the whole blood samples of any case. Of the cases with negative results, Cases 6 and 14 were healthy dogs included as controls and diseased Case 13 appeared not to have a CDV aetiology. The number of foci of viral c.p.e. was less than 10 in the cultures tested for virus isolation by either the cultivation or the ordinary
method. All the positive isolates were identified as CDV by the indirect immunofluorescence test using infected Vero and brain cells. Massive CDV antigen accumulation was demonstrated by specific intense immunofluorescence in the MGC.

The success of virus isolation differed significantly according to the methods and the specimens used as described above. In order to check whether any factors were present which influenced the results of virus isolation, some in vitro biological properties of the isolates and the neutralizing activity of the tissue specimens were compared with special reference to the tissues from which the isolates had originated.

Cytopathic effect and extent of virus release associated with different isolates

The morphology of the c.p.e. in infected Vero cells was compared for DFE virus and isolates derived from brain, lung and CSF of individual cases at early passage levels. The brain-derived isolates induced localized c.p.e. consisting mainly of MGC. Degenerate cells in the centre of areas showing the c.p.e. readily detached from the wall of the culture containers and consequently these areas were readily discernible as discrete plaques without vital staining. The CSF-derived isolates generally induced a similar type of c.p.e. but somewhat smaller MGC, if any, compared with that of the brain-derived isolates. In contrast, the c.p.e. induced by the lung-derived isolates and DFE virus was predominantly composed of rounding cells and eventually spread throughout the cultures (Fig. 5). Pairs of isolates from the brain and the lung of each of Cases 3, 5, 15 and 16 showed this striking contrast in c.p.e. as described above. Two such pairs of isolates from Cases 2 and 8 developed a mixed type of c.p.e., but the brain-derived isolates nevertheless retained the tendency to induce larger MGC than did the lung-derived isolates.

To assess the extent to which the isolates could cause release of infective virus in affected tissues, infective titres of extracellular virus produced in Vero cells infected with viruses derived from different tissues of the same case were assayed and compared. The brain-derived isolates produced less extracellular virus than the lung-derived isolates. Virus release after infection by the isolates from Cases 5, 8 and 15 was greater than that of Cases 2, 3 and 16. No isolate was so productive as DFE virus (Fig. 6).
Properties of encephalitogenic CDV in vivo

Fig. 6. Comparison of virus release from Vero cells infected with DFE virus (■) and isolates from the cerebellum (●) and lung (▲) of (a) Case 2, (b) Case 3, (c) Case 5, (d) Case 8, (e) Case 15 and (f) Case 16.

Table 3. Virus neutralizing titre and virus isolation

<table>
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<th>Case no.</th>
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<th>Lung</th>
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</table>

* Reciprocal NT/virus isolation. NT given as 0 denotes an undetectable level even in undiluted sample.
† NT, Not tested.

Neutralizing activity of tissue homogenates and CSF

The same tissue homogenates and CSF as used for virus isolation were tested for neutralizing activity (NA) to CDV (Table 3). They contained undetectable or low levels of NA if any and their titres roughly correlated with the serum NT titres of the respective cases. Even when brain homogenates prepared from confirmed cases and containing undetectable levels of NA were tested for virus isolation, almost all of them gave negative results. Two cerebellar homogenates, of Cases 3 and 16, from which the infectious virus could be isolated reasonably easily did not
contain a detectable level of NA. Virus was often isolated from lung homogenates in spite of the
presence of a somewhat higher level of NA in them than was found in the corresponding brain
homogenates. However, lung homogenates of Cases 4 and 12, which had a high NA
proportional to the serum NT titre, gave negative results for virus isolation. The presence of NA
in the CSF made direct virus isolation difficult. Among the confirmed cases, only the CSFs
having NA were negative for virus isolation, while those without NA were all positive.

The results described above indicated that the causative virus present in the brain was more
highly cell-associated than the virus in the lung and in in vitro cultures. In order to elucidate the
molecular mechanism by which the virus could remain cell-associated and persist in the brain,
SDS-PAGE analysis of the viral polypeptides was performed on isolate-infected Vero cells and
the affected tissues with special reference to those tissues where the virus had been found to be
harboured.

**SDS-PAGE analysis of viral polypeptides synthesized in infected Vero cells during isotopic
labelling**

Infected Vero cells were radiolabelled in the presence of $^{14}$C-amino acids for 30 min. The
viral polypeptides synthesized during this time were analysed by SDS-PAGE (Fig. 7). Other
investigators have identified eight viral polypeptides in CDV-infected cell lines and designated
them as L, H, P, F0, NP, F1, M and F2 by analogy with measles virus (Hall et al., 1980). Each of
the polypeptides was clearly seen in the lane containing the DFE virus sample included as a
reference and for markers of CDV polypeptides. The SDS-PAGE patterns of the viral
polypeptides of the isolates were diverse because of variation in the amount and mobility of the
P and the H proteins between individual cases. The patterns were similar only when samples
were compared among isolates derived from the same case. The synthesis of NP by the isolates
was as active as that by DFE virus. The ratios of NP synthesis to that of the other viral
polypeptides were very high, whereas synthesis of M was markedly decreased and F0 was
missing. The other viral polypeptides were also relatively decreased in amount.

**SDS-PAGE analysis and radioimmunodetection of viral polypeptides present in infected Vero
cells and affected tissues**

Western blots were made after SDS-PAGE of the lysates prepared from unlabelled cells
simultaneously cultivated with and processed identically to the radiolabelled cells, and then the
total amount of viral polypeptides in the blots was radioimmunologically detected (Fig. 8). As
shown in the lane for DFE virus, all CDV polypeptides could be clearly seen in abundance. In
Vero cells infected with the isolates at the third to the seventh passage levels, the NP, the P and
the L proteins which are located in the inner parts of the CDV virion were present in abundance
and were comparable to those of DFE virus. The SDS-PAGE mobility of P of the isolates varied
from case to case, but was similar when the isolates were derived from the same case. The
amount of H was decreased to a variable extent. F1 was consistently deficient and F0 was
lacking. These decreased viral polypeptides are the structural components which reside on the
surface of the virion and the infected cell. The M protein, which plays a role in viral maturation
(Yoshida et al., 1979), was markedly decreased although it was certainly present. Thus, the SDS-
PAGE patterns of the viral polypeptides visualized by radioimmunodetection were essentially
similar to those obtained by analysing the isotopically labelled cells. The amounts of each of the
viral components in Vero cells infected with the isolates significantly differed from those of DFE
virus. Radioimmunodetection much improved the sensitivity and specificity of detection of the
viral polypeptides present in infected Vero cells, but detected nothing in uninfected cells.

To analyse directly the viral polypeptides present in the affected tissues, this highly sensitive
and specific detection method was also applied to Western blots transferred after separation by
SDS-PAGE (Fig. 9). In the cerebella, NP was detected definitely in Cases 1 to 5, 12, 15 and 16,
faintly in Case 8 and as only a trace in Cases 7 and 9. These results were in good agreement with
those obtained by the immunofluorescence test with the exception of Case 8 which showed a
discrepancy between the intensity of immunofluorescence and the density of the autoradiogram.
Fig 7. SDS-PAGE analysis of CDV polypeptides synthesized in Vero cells infected with isolates. The viral polypeptides were analysed by SDS-PAGE using 5 μl of the lysates prepared from isotopically labelled cells; the slab gels were processed for autoradiography and the autoradiograms were developed after 7 days of exposure. The upper figures above the lanes show the case number. The letters in the middle represent the tissue specimens from which the tested isolates were recovered: cells of cerebellum (C) and brain stem (S), homogenates of cerebellum (Ch) and lung (L), cerebrospinal fluids (F). DFE virus-infected (I) and uninfected Vero cells (U) were included as controls. The lower figures indicate the passage level of the isolates when they were tested. The letters in the centre designate the positions of the polypeptides of DFE virus.
Fig. 8. Analysis of CDV polypeptides in Vero cells infected with the isolates, by SDS-PAGE followed by Western blotting and radi-immunodetection. The viral polypeptides were separated by SDS-PAGE using 5 μl of the lysates of isotope-labelled cultures prepared simultaneously with the cultures and then processed identically. The proteins were radi-immunologically identified. The autoradiograms were developed after 7 days of exposure. The abbreviations for the samples are as for Fig. 7.
Fig. 9. Analysis of CDV polypeptides in the affected tissues of the cases by SDS-PAGE followed by Western blotting and radioimmunodetection. The viral polypeptides were analysed using 10 μl of the tissue lysates as for Fig. 8. Abbreviations for the samples are as for Fig. 7. The figures under I and U indicate the reciprocal dilutions of the lysates prepared from DFE virus-infected (I) and uninfected Vero cells (U). The letters in the centre show the positions of DFE polypeptides.
The H protein was found dimly in Cases 1 and 5, and densely in Case 15. The other viral polypeptides including the third major one, M, could not be recognized. In the lungs, the NP was clearly demonstrated in Cases 2 to 5, 8, 15 and 16, and faintly in Cases 7 and 9, which was also consistent with the results by immunofluorescence. The H protein was detected in Cases 2 (in other experiments), 4, 5, 8 and 15. The amounts of NP and H were much higher in the lungs of Cases 4, 5, 8 and 15 than in the cerebella of the respective cases. The M protein was detected indistinctly but certainly only in Case 15 and its migration was slightly less than that of DFE virus; this result was confirmed by immunoprecipitation (data not shown). This protein was not found even in Cases 4, 5, 8 and 16 which had substantial amounts of NP and H. No viral polypeptide was found in the healthy Cases 6 and 14 or in Case 13 which had been found not to have a CDV aetiology.

**DISCUSSION**

In order to study the mechanism of persistent infection in the CNS, it will be reasonable to investigate protracted cases in which the persistent infection has already become established. Since most of the present cases of canine distemper encephalitis also developed the prodromal symptoms of canine distemper at least a few weeks before the first clinical examination (Fig. 1), they appeared to be clinically different from chronic infections such as ODE and multifocal distemper encephalitis (MDE) (Vandevelde et al., 1980) which suddenly manifest neurological signs without any consecutive prodromal symptoms. ODE involves a diffuse panencephalitis and a varying degree of demyelination involving most cortical areas of the CNS with relative sparing of the cerebellum. In contrast, MDE has severe necrotizing focal lesions characterized by demyelination mostly in the cerebellar white matter. The present cases showed the main features of demyelination and viral inclusion body formation mostly confined to the cerebellar white matter with a paucity of inflammatory response, which have been suggested to be features of chronic lesions (Table 1; Raine, 1976). Thus, the present cases rather resembled MDE but were considered to be more acute cases.

It has been suggested in previous studies by other investigators that the demonstration of IFN in the CSF obtained from dogs more than 20 days after experimental infection with CDV is a valid marker for viral persistence and severity of pathological lesions in the CNS (Tsai et al., 1982), and that an intermediate antibody response modifies the clinical course of the infection, tending to permit chronic infection rather than causing recovery from infection (Krakowka et al., 1975). As almost all of the confirmed cases in the present study retained a low level of IFN-like activity in their CSF more than 20 days post-infection and exhibited a moderate to vigorous antibody response (Table 1), it appears that they had already become persistently infected and were at transitional stages between the acute primary infection and the chronic infection with the serious neurological symptoms approximately 2 months or more post-infection.

A point of as much importance as the subjects used in the present study is not how long the diseases ran their clinical courses after establishment of the persistent infection, but is whether the persistent infection had been due to certain interactions between CDV and its infected cells. Although the present cases were not necessarily suitable for the intended purposes from clinical and pathological points of view, they may serve for study of the underlying conditions leading to persistent infection, through virological approaches which can directly elucidate the interactions between the virus and its infected cells which precede the inflammatory responses.

The cultivation methods gave markedly better results for virus isolation from the brain compared to the ordinary method using homogenates from the same case (Table 2). CDV was rarely isolated from homogenates of brains even from preparations containing a great number of immunofluorescent cells and little NA, suggesting that CDV remained cell-associated in situ under certain restrictive conditions imposed by the host. CDV was nevertheless recovered from two out of nine cerebellar homogenates prepared from confirmed cases. Although these results resemble the findings that only cultivation methods make it possible to isolate SSPE viruses, the degree of cell association of CDV is less stringent. In the affected brains of SSPE patients, the P and H proteins of MV are much decreased and their quantities fluctuate case by case. The M is missing, whereas NP is consistently found (Hall & Choppin, 1981). Recently Haase et al. (1985)
investigated replication of MV in the brains of SSPE patients in the early and late stages of the disease, and showed that early in the course there was an overall repression of the synthesis and expression of the genome, and that later a more specific block of the synthesis occurred. This is thought to explain the cell association of the causative virus in situ and the difficulty in virus isolation from brain homogenates. The present in vivo study demonstrated that in addition to the overall repression of viral polypeptide synthesis, viral components other than NP are much decreased (Fig. 9), which was similar to the situation in the SSPE cases. Thus, the difficulty in virus isolation from brain homogenates of the present cases can be understood by analogy to SSPE, and the present cases look promising as a useful animal model to study in vivo mechanisms controlling persistent infection by morbilliviruses in the brain of chronic cases.

The rate of successful virus isolation from brain homogenates was significantly lower than that from lung homogenates (Table 2). What causes this was investigated by comparing some in vivo and in vitro properties of CDV and the NA of the tissue homogenates with special reference to the tissues where the virus had been harboured. The in vivo study detected only the NP and H proteins and showed a slight decrease in the quantity of these antigens in the brain tissues. The other viral polypeptides were below the level for detection in both these tissues (Fig. 9). The isolation rates are partially reflected by a quantitative difference in the SDS–PAGE patterns of detectable viral polypeptides present in brain and lung of the same individual, but are not necessarily explained by absence of the M (which is involved in virus maturation) except from Case 15. Because the number of foci of c.p.e. observed in cultures inoculated with lung homogenate was generally less than 10, the level of productive infection yielding complete virus in the lung may be too low to allow detection of characteristic SDS–PAGE patterns. Thus, the in vivo study demonstrates the above quantitative difference to be due to a high level of non-productive infection by the virus which is cell-associated in both the tissues. On the other hand, whereas lung homogenates contained somewhat higher NA than brain homogenates, the former gave far better results for virus isolation (Table 3).

At low passage levels, the brain- and the lung-derived isolates induced different types of c.p.e. (Fig. 5) and produced a considerable amount of extracellular virus, although the brain isolates were less productive (Fig. 6). In parallel with this conversion to productive infection, the synthesis of some viral polypeptides, whose synthesis had been repressed in vivo, was restored to some extent and their SDS–PAGE patterns were indistinguishable only when the isolates had been derived from the same individual (Fig. 7 and 8). The in vitro study gave results suitable for explaining the difference in the degree of cell association between the isolates and DFE virus, but not for this phenomenon. Passage of the isolates in vitro appeared to induce their adaptation to a single cell line (Vero cells), and it resulted in reduction of their unique in vivo property of cell association and hence in deviation from the in vivo state. These results also suggest that the phenotype of the virus had been reversibly modified under the different conditions for virus replication in the host tissue in which the progeny of an initially infecting virus had resided.

While it must be considered a possibility that the overall repression of in vivo viral polypeptide synthesis as visualized by radioimmunodetection (Fig. 8 and 9) was an artefact which arose from an antigenic difference between the causative CDV and DFE virus, the observations that substantial amounts of all the viral polypeptides were detected in the lung of Case 15 by radioimmunodetection (Fig. 9) and that similar results of their in vitro synthesis were obtained by isotopic labelling (Fig. 7) and radioimmunodetection (Fig. 8) make this unlikely.

Virus was frequently recovered from the CSF and whether virus isolation succeeded or not was antithetical to the presence of neutralizing antibody to CDV in them (Table 3), suggesting that the virus present in the CSF is cell-free and differed from the virus which remains cell-associated in the brain parenchyma. These viruses appear to be derived from infected cells of different origins.

An overall repression of viral polypeptide synthesis was also found in combinations of brain cell lines (118 MGC glioblastoma and KG-1 oligodendroma) and the Mantooth strain of SSPE virus (Sakaguchi et al., 1984). A drastic reduction in M protein synthesis occurred in Vero cells infected with the Niigata-1 strain of SSPE virus due to defective translation (Carter et al., 1983). Cyclic AMP treatment of a human neural cell line (IMR-32 neuroblastoma) infected with the
Edmonston strain of MV caused overall repression by inhibition of transcription (Yoshikawa & Yamanouchi, 1984). In order to define further the mode of infection in the present cases, it will be necessary to determine whether translational or transcriptional inhibition is responsible for repression.

It has been stressed that two conditions which allow infected cells to survive for long periods must be met if viral persistence is to occur: (i) prevention of the direct destruction of infected cells due to lytic infection and (ii) escape of the infected cells from the host's immune surveillance (Haase et al., 1981). Oldstone & Fujinami (1982) proposed a hypothetical mechanism to explain the second criterion, in which the presence of specific antibody during infection induces antigenic modulation and/or redistributes the antigens so as to reduce their expression on the infected cell surface which therefore ceases to be the target of the cytotoxic response. The overall repression as well as the relative decrease in the viral surface proteins demonstrated in vivo in the present study appear to satisfy both the above two conditions and thus may be responsible for the viral persistence in these immune cases. However, restricted viral replication as observed in this study is not always exclusively caused by a particular defective mutant, but may be a general phenomenon occurring in the brains of transitional cases infected with an unmodified virus and having a full immune response.

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REFERENCES


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