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Pathogenesis of Borna Disease in Rats: Evidence that Intra-Axonal Spread Is the Major Route for Virus Dissemination and the Determinant for Disease Incubation

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Borna disease virus is an uncharacterized agent that causes sporadic but fatal neurological disease in horses and sheep in Europe. Studies of the infection in rats have shown that the agent has a strict tropism for neural tissues, in which it persists indefinitely. Inoculated rats developed encephalitis after an incubation period of 17 to 90 days. This report shows that the incubation period is the time required for transport of the agent in dendritic-axonal processes from the site of inoculation to the hippocampus. The immune responses to the agent had no effect on replication or transport of the virus. The neural conduit to the brain was proven by intranasal inoculation of virus that resulted in rapid transport of the agent via olfactory nerves to the hippocampus and in development of disease in 20 days. Virus inoculation into the feet resulted in spread along nerve fibers from neuron to neuron. There was sequential replication in neurons of the dorsal root ganglia adjacent to the lumbar spinal cord, the gracilis nucleus in the medulla, and pyramidal cells in the cerebral cortex, followed by infection of the hippocampal neurons and onset of disease. This progression required 50 to 60 days. The exclusiveness of the neural conduit was proven by failure to cause infection after injection of the virus intravenously or into the feet of neurectomized rats.

Borna disease is a sporadic but fatal infectious neurological disease of horses and sheep in eastern Europe. Experimentally, the etiologic agent of this disease is pathogenic for animals belonging to an unusually wide range of species, extending from birds to mammals, and, among the latter, from rodents to primates (8, 14–18, 23–25). Infected animals develop chronic neurological disease characterized by striking abnormalities in behavior. Behavior varies among different species of host animals; in tupaias (animals that are used routinely in behavioral studies), disease is manifest by hyperactivity and aberrant social interactions with other members of the group (23). In contrast, inoculated rats develop a unique biphasic disease prefaced by an incubation period of several weeks which is followed by hyperactivity and aggression, with a later stage characterized by permanent listlessness and eating disorders (19). Clinical disease is caused by mononuclear inflammatory cells that proliferate in the brain in response to a high level of expression of Borna disease virus (BDV) antigen in cells in the neuropil (18).

The etiologic agent of Borna disease is uncharacterized but it has the general properties of an enveloped virus. The agent is sensitive to lipid solvents, proteolytic enzymes, and irradiation with UV light (C. S. Duchala, unpublished data). However, its nucleic acid has not been identified, and the agent has never been visualized in the electron microscope (1, 2, 10). The agent replicates preferentially in nerve cells both in the animal and in cell culture (7, 19). Infectivity is measured either by inoculation of dilutions of test material intracerebrally (i.c.) into rats or by inoculation into cultures of fetal brain (from rabbits or rats) (7). Infectivity endpoints in the animal assay are determined by development of characteristic disease, production of antibodies to a BDV-specific protein, and recovery of virus from neural tissues. The BDV antigen is easily detected immunohistochemically in sections of tissues from affected animals (4, 15, 22). BDV does not cause cytopathic effects in cell culture, but infected cultures develop BDV-specific antigen that can be stained immunohistochemically with antibodies from any animal with the disease (7). Antigen purified from cell culture is not infectious. It has a molecular size of 38 kilodaltons, and polyclonal and monoclonal antibodies induced by this protein do not neutralize infectivity (5).

The relationship between the infectious particle of BDV and the antigen that is produced during replication of the agent is not completely understood. The antigen is probably encoded by the agent because all animals with naturally or experimentally induced Borna disease develop the antigen in tissues (4). Further, such animals produce antibodies that bind with similar affinity to antigen produced in infected tissue cultures or in tissues of any other animal with the disease. In infected cell cultures, this antigen is visualized as aggregates within the nucleus and diffusely in cytoplasm. In nerve tissues, however, large amounts of the antigen occur in the nuclei of neurons and in the cytoplasm of their dendritic and axonal processes. When stained immunohistochemically, this dense concentration of antigen provides a clear anatomical delineation of these cells and their processes in tissues (10, 15).

Nearly all previous studies on Borna disease had been performed in laboratories in the Federal Republic of Germany, where infection in horses still occurs naturally (4). The virus from horses is virulent for rabbits (16). Infectious homogenate of rabbit brain is highly neurotropic for adult rats, although in neonatally inoculated rats the virus appears

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to replicate in other tissues (6). The neurotropism of the virus for adult rats does not require adaptation by multiple passages in these animals (8, 20). We have recently established the disease model in rats in our laboratories, under special permission from the U.S. Department of Agriculture, using infectious brain homogenate kindly provided by R. Rott from the Justus Liebig University, Giessen, Federal Republic of Germany. We have confirmed that BDV has a selective tropism for cells in nervous tissues and that it causes biphasic disease preceded by a long incubation period. In this report, we have focused on the mechanism of the incubation period of the disease. We show that it is variable and that the variability is determined by the obligatory requirement of the agent for neuronal cells for both replication and dissemination. The agent does not replicate in nonneural tissues. Onset of disease occurs only after the agent has begun to replicate in neurons in the CA3 and CA4 regions of the hippocampus. The incubation period of the disease seems to be the time required for the agent to be transported in neural processes and for it to replicate in the series of neurons which link the site of entry of the virus to neurons of the hippocampus.

MATERIALS AND METHODS

BDV stock. Homogenate of brain from an experimentally inoculated rat with Borna disease was kindly provided by R. Rott, Giessen, Federal Republic of Germany. The material was inoculated i.c. into 1-day-old Lewis rats which were killed 3 weeks later. Brains were removed aseptically and homogenized into a 20% suspension (wt/vol) in minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) containing 2% fetal bovine serum (FBS). The suspension was prepared by Dounce homogenization followed by brief pulses of ultrasonic treatment. The material was clarified by centrifugation at 2,000 × *g* for 10 min, and then it was distributed into vials and frozen at −70°C.

Infectivity assays. Infectivity in tissue homogenates was determined by immunofluorescence of BDV-specific antigens in inoculated cultures of fetal rabbit brain (FRB). Brains from 16- to 18-day-old rabbit fetuses (Bunnyville, Littlestown, Pa.) were minced with scalpels and washed free of meninges and choroid plexus. The tissue fragments were explanted into tissue culture flasks in Dulbecco MEM (GIBCO) supplemented with 20% FBS and then incubated at 37°C in 5% CO_2. Cellular outgrowths were expanded by one subcultivation. Monolayers of these cells were dissociated with trypsin, suspended in growth medium (Dulbecco MEM plus 20% FBS) with 7% dimethyl sulfoxide, and frozen in liquid nitrogen. Ampoules of frozen cells were thawed as needed and expanded through two subcultures before being seeded into eight-well chamber slide preparations (Lab-Tek; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) as previously reported (7). Cells in duplicate chambers were inoculated with 0.5 ml of maintenance medium (Dulbecco MEM plus 2% FBS) containing 10-fold dilutions of infectious tissue homogenates, and the cultures were incubated for 10 days at 37°C in 5% CO_2. The chambers were then dismantled; and the cultures were rinsed in phosphate-buffered saline, fixed in acetone, and processed for indirect immunofluorescence with serum from a rabbit with experimentally induced Borna disease. Fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin (DAKO, Burlingame, Calif.) was then applied to the slides. The slides were examined with a fluorescence microscope for the presence of the punctate granules of intranuclear fluorescence that are typical of the BDV antigen (7). Each assay result was normalized to an internal control, which used a standard of brain homogenate containing a known infectivity titer.

Antibody assays. All inoculated rats that developed clinical Borna disease produced antibodies that stained BDV antigens in MDCK cell cultures, a persistently infected cell line developed in Giessen, Federal Republic of Germany (7). The antibody titer in these sera was determined by indirect immunofluorescence, using twofold dilutions in the primary reaction followed by fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin (ICN Immunobiologicals, Lisle, Ill.). Some of these sera were tested for neutralizing antibodies by incubating twofold dilutions with an equivalent volume of infected rat brain homogenates containing 10^2 50% tissue culture infectious doses ($TCID_{50}$) of stock BDV at 37°C for 30 min before inoculation into FRB cultures. These cultures were examined 7 days later to determine whether antibodies had prevented development of the BDV antigen. Preincubated virus (10^4 $TCID_{50}$)-antibody (1:10 dilution) mixtures were also inoculated intranasally (i.n.) and intracranially into rats (see below) to determine whether the antibodies would protect rats against the development of disease.

Immunization with UV-irradiated BDV. Stock virus was exposed to UV irradiation for 1 h, emulsified with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), and injected intradermally into five Lewis rats. The rats were injected three more times during the next 2 months with the UV-treated stock suspension emulsified in Freund incomplete adjuvant (Difco). Two immunized rats were killed, and the tissues were examined immunohistochemically for BDV antigen to ensure the absence of viral infection. Two immunized rats and several control rats were inoculated i.n. with 10^4 $TCID_{50}$ and observed for 100 days. One immunized rat was also kept for long-term observation.

Rats. Throughout these studies 5- to 6-week-old male inbred Lewis rats, purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., were used. Rats were anesthetized and inoculated with BDV via different routes. Metofane inhalation anesthesia (Pittman-Moore, Inc., Washington Crossing, N.J.) was used for i.c., subcutaneous (s.c.), intravenous (i.v.), and oral inoculations. i.c. inoculations were performed by direct injection of 50 μl of material into the brain with a 26-gauge, 0.5-inch (1.27-cm) needle. A similar injection was administered s.c. in the thigh. i.v. inoculations were given in one of the tail veins. Pediatric infusion catheters with 25-gauge needles were used for entry into the veins. The tubing of the catheter was filled with normal saline, and injection of virus material was administered at this site only when gentle aspiration produced a flashback of blood, indicating that the needle was in the bloodstream. Then, 200 μl of infectious material (10^6 $TCID_{50}$ of BDV per ml) was injected into the catheter, and this was chased with 200 to 300 μl of normal saline before withdrawing the needle. This precaution was taken to reduce the chance of injecting the material s.c. or into the nerve that runs parallel to the vein. Oral inoculation was performed after gastric intubation.

Inoculations via the i.n. and footpad routes and surgical neurectomies were performed on rats anesthetized by intraperitoneal injections of 400 mg of chloral hydrate (Sigma Chemical Co., St. Louis, Mo.) per kg. The i.n. inoculation was performed by direct deposit of the inoculum (infectious material or virus-antibody mixtures) on the nasal mucosa, thus obviating trauma by the needle. Inoculation into the

footpad was performed by placing a drop of infectious material on the ball of the hind foot and using multiple needle pricks with a 26-gauge needle to introduce the material into the dermal-s.c. area (12). The excess material was then wiped from the surface of the skin with an alcohol swab.

Neurectomy. The sciatic nerve was exposed by a skin incision on the lateral aspect of the thigh of one leg, and a 1-cm-long section of the nerve was resected. The incision was then closed with surgical clips. Virus was injected into the footpad of that (ipsilateral) leg or the other (contralateral) leg 2 days later. Another series of rats underwent sciatic nerve resection at 1, 2, 3, and 5 days after inoculation of virus into the hind-limb footpad.

Intramuscular route. The gastrocnemius muscle of the hind limb was exposed through a 0.5-cm incision. A 26-gauge needle was used to inject 0.2 ml of viral stock (mixed with an inert carbon marker) into the belly of the muscle. The incision was closed as described above. At various intervals after inoculation, rats were processed as follows: muscle from two rats was frozen in OCT medium (Miles Laboratories, Inc., Elkhart, Ind.) on dry ice, cut into 8-μm sections, and air dried. Each section was examined for BDV antigen by the indirect fluorescence assay described above. Muscles from two other rats were homogenized and examined for infectious virus by the FRB assay mentioned above.

Morphological studies. Rats were anesthetized with chloral hydrate and perfused through the heart with 250 ml of cold normal saline followed by 150 ml of cold 4% paraformaldehyde administered by a perfusion pump (Manostat, New York, N.Y.). Various tissues were then dissected out and processed before being embedded in paraffin. Tissue sections were then processed and stained with hematoxylin and eosin. Duplicate slides were processed for identification of viral antigens with the use of the avidin-biotin immunoperoxidase assay (Vectastain; Vector Laboratories, Burlingame, Calif.). Rabbit antibodies to BDV antigen were used as the primary antibodies. This procedure was followed by application of biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories) and later by the avidin-biotin complex.

RESULTS

Disease in rats. Assay of infectivity of our stock virus showed an infectivity titer of 10^6 $TCID_{50}$ in the immunofluorescence test in FRB cultures and a similar titer by induction of disease in 6-week-old rats. Therefore, the term $TCID_{50}$ is used to quantitate infectivity either in cell culture or in animals. Infected rats developed the unique signs of disease described previously (19). The disease followed a predictable course, with a transient acute phase followed by a prolonged chronic phase that were both characterized by dramatic changes in behavior. The early acute signs of disease were characterized by sudden onset of ataxia and exaggerated responses to stimuli, as evidenced by frantic running and leaping when stimulated by sudden noise. During this stage, some of the rats responded to handling with frenzied struggling and biting. Ataxia worsened in some animals, and many of these died. Rats that were only mildly ataxic developed a wasting syndrome at 2 to 3 weeks after onset of the acute signs. They ceased normal grooming behavior. Further, the frenzied activity that had characterized their behavior earlier changed to extreme listnessness, a behavior pattern they maintained for the rest of their lives. Many became blind. They continued to show spastic responses to stimuli. A few of these inactive animals became hugely obese. Approximately 80% of the animals died during the 6-month study.

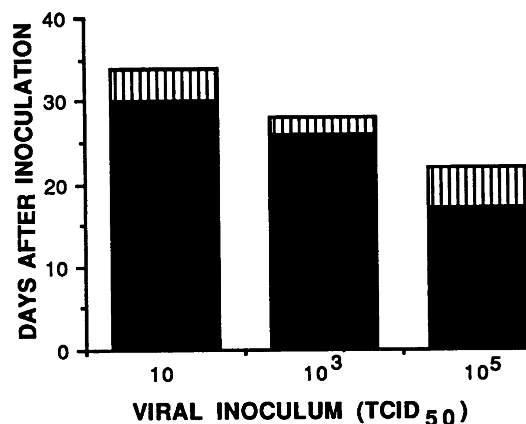


FIG. 1. Incubation period of Borna disease after i.c. inoculation. Three groups of five male, 5-week-old Lewis rats were injected intracranially with either 10, 10^3, or 10^5 $TCID_{50}$ of BDV. These groups were observed daily for signs of Borna disease. The incubation period ended when the first rat in each group showed signs of acute disease, such as agitation or aggression (■). The time required for all rats to exhibit signs of disease (range in length of incubation period) is indicated (▥).

Variation in the incubation period of Borna disease. The clinical pattern of disease described above was seen in all subsequent study groups (5 to 10 rats per group) described below. The sequence of events leading to chronic disease was always predictable. The major difference among various groups, however, was the incubation period of the disease. This varied considerably, extending from 17 to 90 days after inoculation. The studies described below were aimed at understanding the mechanism of this variation.

We first determined whether the incubation period of the disease could be correlated with the number of infectious units in the inoculum by inoculating groups of 5 to 10 rats with dilutions of the stock virus preparation containing 10^5, 10^3, 10, and approximately 1 $TCID_{50}$. There was a strong correlation between the two parameters (Fig. 1). An inoculum containing 10^5 $TCID_{50}$ caused disease on day 17, 10^3 $TCID_{50}$ caused disease on day 28, and 10 $TCID_{50}$ caused disease on day 32. Within 4 days after the first animal in each group became ill, all animals in that group had developed disease. The possibility that virus may have spread from animal to animal was ruled out in control experiments in which 10 uninoculated rats housed in the same cage with infected rats for several months did not seroconvert or become ill (unpublished data). A further consistency among the three groups was that the first mortality occurred within a week after clinical signs appeared. Thus, once the incubation period of the disease ended, the disease progressed at a constant rate regardless of the number of infectious units in the inoculum. An inoculum containing between 1 and 10 $TCID_{50}$ had a more variable effect. All rats developed disease by day 90, but the incubation period extended from 35 to 90 days. Examination of sera from rats in different groups showed that all animals that developed disease produced antibodies to BDV antigen. The titers extended from 1:80 to 1:2,560. Animals inoculated with a 10-fold-higher dilution than 1 $TCID_{50}$ failed to develop disease. The animals did not produce antibodies, and infectious virus could not be recovered from their brain homogenates. Homogenates of

TABLE 1. Lack of protection by anti-BDV antibodies in rats challenged with BDV[a]

Rats[a]	Antibody titer	Disease onset (day)
Normal	<1:10	27
Immunized[b]	1:320	30

[a] Rats received a challenge dosage i.n. of 10^4 $TCID_{50}$ of BDV.
[b] Rats were previously injected with UV-inactivated BDV.

TABLE 2. Lack of neutralization or protection by anti-BDV antibodies

Test system[a]	Parameter	Neutralization titer
Tissue culture	Development of antigen	<1:10
Rats inoculated i.c.	Disease	<1:10
Rats inoculated i.n.	Disease	<1:10

[a] BDV was preincubated with doubling dilutions of antiserum (immunofluorescence assay titer of 1:640) at 37°C for 30 min before being inoculated into tissue culture (10^2 $TCID_{50}$) or rats (10^4 $TCID_{50}$).

brains from rats with disease had approximately 10^6 $TCID_{50}$ per g. Thus, clinical disease correlated with productive virus replication in brain and with development of specific antibodies in serum, but the time of onset of disease depended on the number of infectious units in the inoculum.

The remarkably close correlation between the incubation period of the disease and the dose of virus suggested that the pathogenesis of this infection might be influenced by two independent host-virus interactions. First, the observation that all cases of infection eventually led to disease suggested that infection always resulted in persistence of the virus and that immunizing infections did not occur. Second, the delay in onset of disease resulting from a low dose of virus in the inoculum suggested that a minimal threshold of virus replication was required to trigger the disease process. Both of these concepts were examined experimentally.

Immunization experiments. In this series of experiments, we sought to determine whether the humoral arm of the immune response to BDV had any effect on the pathogenesis of infection. We inactivated the infectivity in a 2-ml stock virus preparation with UV irradiation (evaluated by failure to infect FRB cultures) and used this material to immunize five rats. These animals developed antibodies (titers of 1:320 to 1:640) to BDV antigens, as measured by immunofluorescence assay on BDV-infected MDCK cell monolayers. Of these animals, two were inoculated i.n. with 10^4 $TCID_{50}$. Further, a virus suspension containing 10^4 $TCID_{50}$ was preincubated with serum from an experimentally infected rat with an antibody titer of 1:640 for 30 min at 37°C; the suspension was then inoculated into four normal rats i.c. and four normal rats i.n. Control rats were inoculated with the same amount of virus diluted with nonimmune rat serum by similar routes. The results illustrated in Table 1 showed that prior immunization of rats with BDV antigens did not protect against infection (demonstrated by BDV antigen formation in brain) or disease after i.n. challenge and had no effect on the incubation period of the disease. Preincubation of immune serum with virus did not protect FRB cultures against infection or rats against disease (Table 2). Thus, antibodies to BDV antigens seem to have negligible biological effects on the agent and have no apparent role in prevention of infection or in acceleration of the onset of disease.

Time for development of BDV antigen and infectivity in the nervous system. To determine whether a minimum threshold of virus replication was essential for initiating onset of disease, we inoculated two groups of rats i.c. with different concentrations of BDV. A total of 50 animals were inoculated with 10^5 $TCID_{50}$, and 20 animals were inoculated with 10^2 $TCID_{50}$. Animals in each group were killed at sequential intervals, and tissues were examined for infectivity, histopathologic changes, and viral antigen. Blood was also collected for antibody determinations.

Rats inoculated with 10^5 $TCID_{50}$ were examined in the following order. Homologous tissues pooled from two rats were examined for virus content every second day for the first 3 weeks after inoculation, tissues from two rats were examined at weekly intervals for the next month, and tissues from two rats were examined at intervals of 2 to 3 weeks until the fifth month. The first animal became ill on day 17, and by 22 days after inoculation all animals developed clinical disease. Infectious virus was first detected in the brain on day 8 and in cervical and lumbar regions of the spinal cord by day 11. In the brain, virus titers reached peak values on day 21, with 10^7 $TCID_{50}$ per g (Fig. 2). There was a gradual decline during the following weeks, but the titer at day 160 was still 10^4 $TCID_{50}$ per g. Cervical and lumbar regions of the spinal cord produced similar amounts of infectious virus. Samples of salivary glands, kidneys, spleens, cervical lymph nodes, and lungs from three rats were examined at regular intervals throughout the infection. No infectivity was ever found in these homogenates.

Small amounts of viral antigen were first detected on day 9 in a few neurons in the CA3 region of the hippocampus and layers 4 and 5 of the frontal cortex of the cerebral hemispheres. By day 15 the concentration of staining of antigen had become more intense and antigen had spread to most of the hippocampal neurons and the hypothalamus. These findings coincided with the onset of clinical disease. Antigen was also present at this time in other regions of the brain and included neuronal groups in the deep cerebellar nuclei and

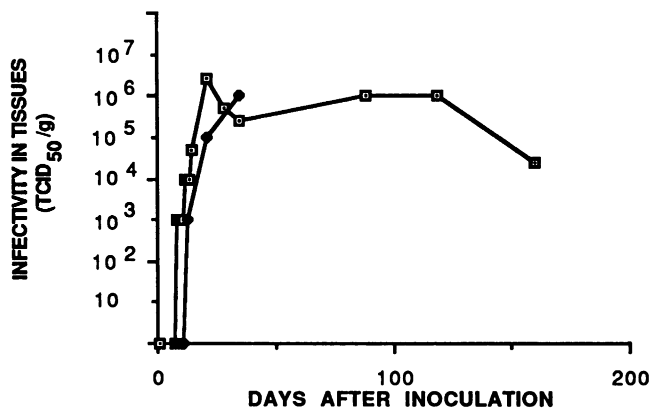


FIG. 2. Rate of replication of BDV in the central nervous systems of 70 male, 5-week-old Lewis rats injected i.c. with 10^5 $TCID_{50}$ (□) or 10^2 $TCID_{50}$ (■). Two rats at a time were killed at various times until 160 days, and homogenates of tissue samples were measured for infectivity by immunofluorescence in inoculated FRB cultures.

nuclei in the brain stem, the trigeminal ganglia, and the dorsal root ganglia of the spinal cord in both cervical and lumbar regions. By day 20 antigen was detected in the large motor neurons in the ventral horn of the spinal cord. Dense concentrations of antigen were found in individual fibers of the sciatic nerve as well as in the cranial nerves at this time. Beyond 23 days after inoculation the antigen had become widely disseminated in the brain, spinal cord, spinal ganglia, and peripheral nerves and also in sympathetic ganglia in the viscera. At infrequent intervals antigen was also detected in the parasympathetic nuclei in the neuronal plexuses of the intestine. In nearly all cases, antigen was localized in both nuclei and cytoplasm of cell bodies and vividly outlined dendritic processes and axons. Beyond day 23, antigen was also present in cells morphologically resembling astrocytes. Sections examined at 100 days and beyond after inoculation showed a great loss of cells from the cerebral hemispheres. Viral antigen was located in scattered cells throughout the brain, but the intensity of staining had become low. Further, in nearly all the cells, antigen was located mainly in the nuclei. No antigen was detected in nonneural tissues, including the pancreas, salivary glands, mucosa of the intestine, or lymph nodes. Thus, inoculated rats had developed a persistent productive infection that was confined to the nervous system (Fig. 3).

Histopathologic changes consisted essentially of dense infiltration of mononuclear cells in the meninges, around the blood vessels, and in the parenchyma of the brain and spinal cord. These inflammatory cells had also infiltrated around cell bodies in the ganglia and in between nerve fibers in the dorsal column of the spinal cord and the sciatic nerve. The inflammatory reaction in the brain coincided with the onset of acute disease. Degeneration of parenchymal cells in the brain occurred after the influx of inflammatory cells and culminated in widespread cell loss in the grey matter of the brain and spinal cord. There was also severe loss of neurons in the cranial and spinal ganglia. Wallerian degeneration of nerve fibers in the sciatic nerve and in the dorsal columns of the spinal cord accompanied the loss of infected neurons in the dorsal root ganglia. At approximately 40 days after the onset of disease, the severity of the inflammation subsided. Loss of cells in the brain was evidenced by thinning of the cortical mantle, with concomitant enlargement of the lateral ventricles, and in some cases by prominent hydrocephalus.

Antibodies to BDV antigens were first detected on day 20 and coincided with the accumulation of inflammatory cells around neuronal and glial cells. Titers increased from 1:20 on day 20 to greater than 1:2,560 by 6 weeks after inoculation.

In summary, in rats inoculated i.c. with 10^5 $TCID_{50}$ of BDV, infectious virus was detected first in brain homogenates on day 8 and titers reached peak values (10^7 $TCID_{50}$ per g) by day 21. Infectious virus was present in all subsequent samples of central nervous system (CNS) tissue up to the last sampling on day 160 (10^4 $TCID_{50}$ per g). Viral antigen was first detected on day 9 in a few neurons of the CA3 and CA4 regions of the hippocampus and in layers 4 and 5 of the frontal cortex. Antigen increased in intensity during the following week and had disseminated throughout the brain, spinal cord, and cranial and spinal ganglia by day 23. Serum antibodies first appeared on day 20 and persisted to the end of the experiment. Inflammatory cells were first detected on day 18 and reached peak intensity by days 30 to 40. The aggressive phase of clinical disease was first seen on day 17, reached peak intensity by day 25, and gradually gave way to the second phase (listlessness and hypoactivity) by day 32.

i.c. inoculation of rats with 10^2 $TCID_{50}$ recapitulated a sequence of events similar to those caused by inoculation with 10^5 $TCID_{50}$ except for a delay in all parameters of the infection, i.e., viral infectivity, viral antigen, antibody, histological changes, and clinical disease. Thus, infectivity was first detected in the brain on day 13 and reached peak values (10^6 $TCID_{50}$ per g) by day 33. BDV antigen first appeared on day 11 in a sequence similar to that described for the larger inoculum. Encephalitis appeared on day 28, coinciding with the onset of acute disease.

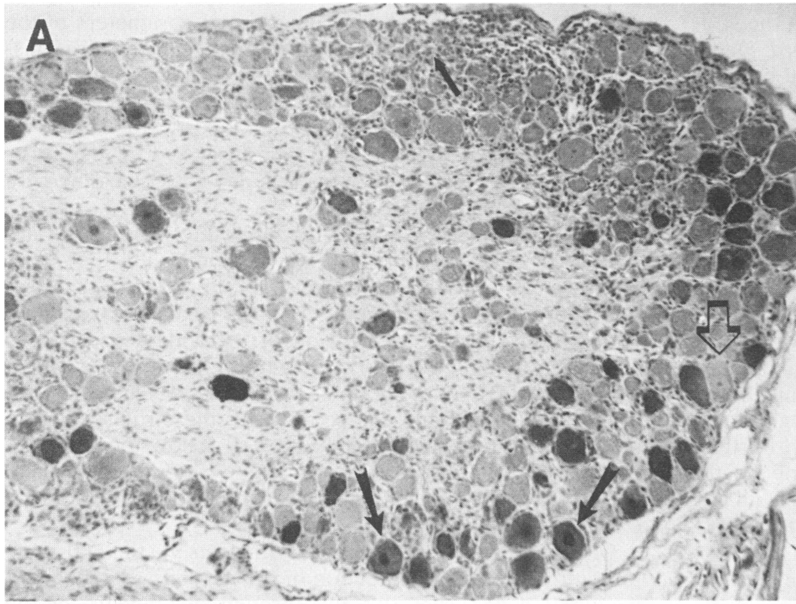
This experiment revealed virus-host interactions that are unique to this infection. First, it confirmed the strict neurotropism of BDV described in earlier reports (4). Second, it provided evidence that the onset of disease coincided with a high level of expression of viral antigens and the attendant inflammatory response in the nervous system. Third, the incubation period of disease was variable but followed a predictable course. Fourth, the virus seemed to disseminate along neural pathways. To follow the transit of the agent in the nervous system, we investigated the neuroinvasive potential of the virus after inoculation into several extraneural sites.

Oral route. We inoculated rats with virus via the oral route to determine whether infection in the nervous system could be initiated by virus invasion through the mucosa of the gastrointestinal tract. This seemed particularly relevant because viral antigens were found in the parasympathetic ganglion cells in the intestinal neural plexuses of rats with disease. Five Lewis rats were inoculated with undiluted stock virus introduced into the stomach with a gastric cannula. The animals neither developed clinical disease nor showed evidence of infection (Table 3). There was no infectious virus in nervous tissue and no BDV antigen in paraformaldehyde-fixed tissue sections, and anti-BDV antibodies did not develop in the serum of animals after 100 days of observation. The experiment was terminated at this time, with the conclusion that infection of the nervous system via this route would be inefficient.

s.c. route. Inoculation of stock virus preparation s.c. in the thigh of two rats caused disease after an incubation period of 55 days (Table 3). This established the feasibility of successfully infecting the CNS from a peripheral site.

i.v. route. To investigate the role of blood as a conduit to the brain, we performed i.v. injections, using the precautions outlined in Materials and Methods to ensure deposition of the virus in the bloodstream. Eight rats were inoculated at weekly intervals for 3 weeks with 0.2 ml of undiluted stock virus. None of the animals became ill during the following 60 days. During the following month, four of the animals developed antibodies with titers of 1:80. However, these antibodies did not neutralize virus infectivity (data not shown). The animals appeared clinically normal. Two animals were killed and examined for possible occult infection, but neither viral infectivity nor viral antigen was found in the brain (data not shown). This antibody response may have resulted from the antigenic stimulation produced by the three i.v. inoculations of brain homogenate. Only three of eight rats developed disease over a 90-day observation period, so this route of inoculation was not efficacious in causing infection in the brain despite repeated inoculations with massive doses of infectious material.

i.n. route. Five rats were inoculated i.n. with infectious brain homogenate. All five animals became acutely ill between 18 and 21 days after inoculation. Infectious virus (10^5 $TCID_{50}$ per g) was present in homogenates of brain, and dense concentrations of viral antigen were present in neu-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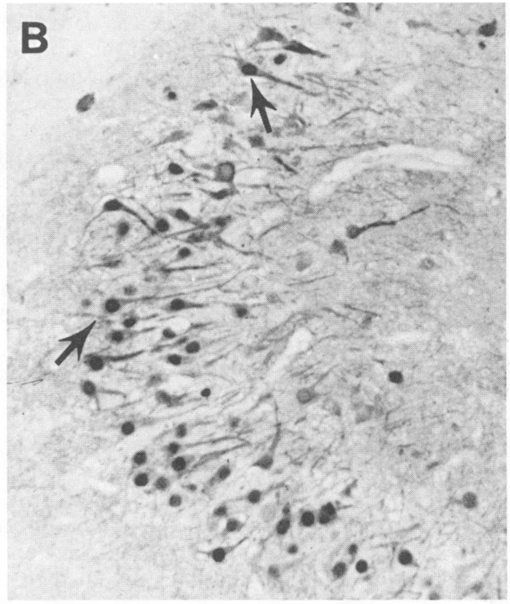
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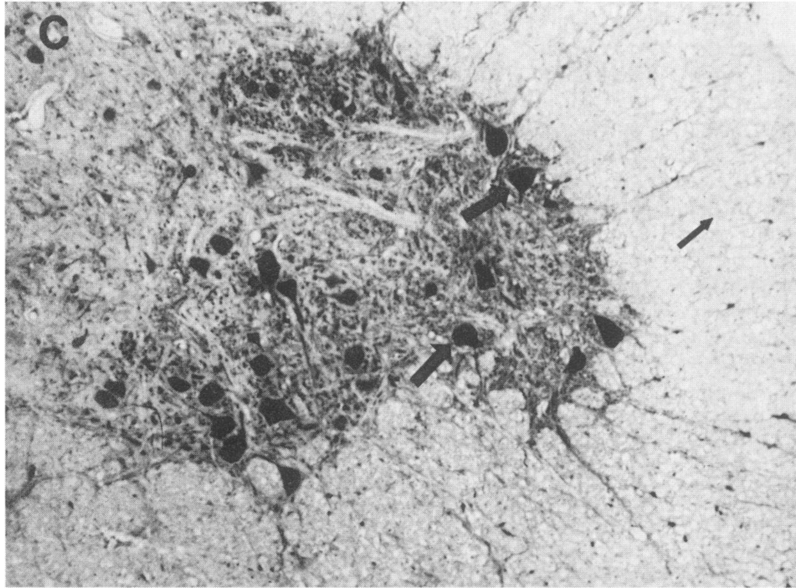
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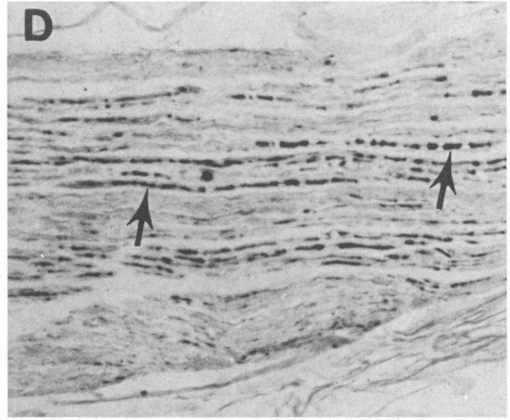
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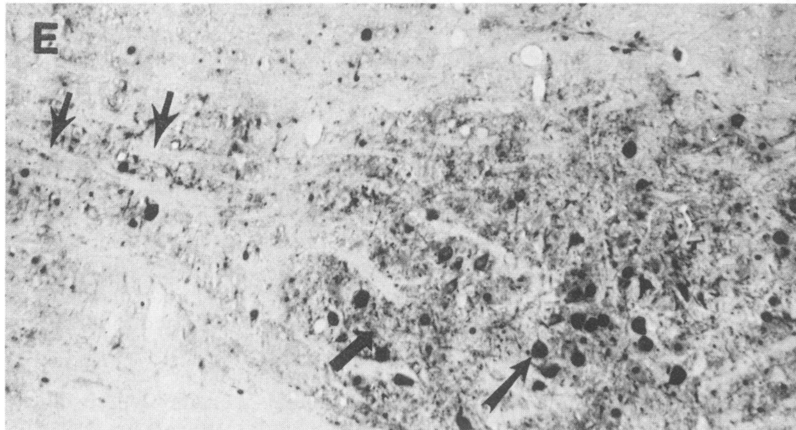
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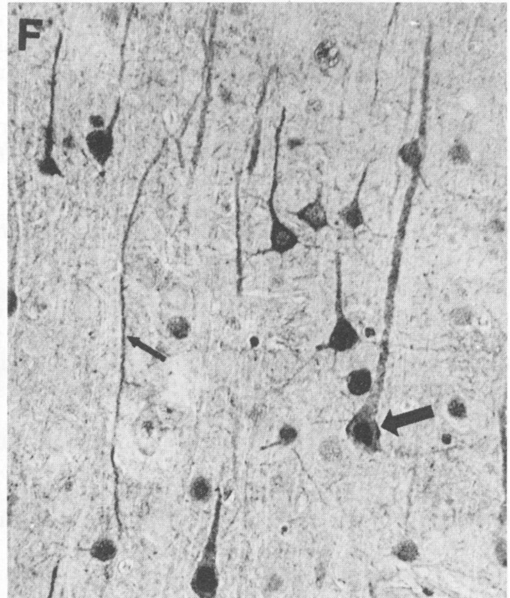
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TABLE 3. Variation in incubation period of disease after a single virus inoculation (10^5 to 10^6 $TCID_{50}$) by different routes

Inoculation route	Incubation period (days)	% Animals infected (no. infected/no. inoculated)
Intracranial	17	100 (5/5)
i.n.	21	100 (4/4)
i.v.[a]	54	38 (3/8)
s.c.	55	100 (2/2)
Footpad, sciatic nerve intact	47	100 (12/12)
Footpad, sciatic nerve transected	92	8 (1/13)
Contralateral foot	60	100 (4/4)
Gastric intubation	No disease	0 (0/5)

[a] Three injections of undiluted stock virus at biweekly intervals.

rons in the frontal cortex and in the hippocampus. This route of inoculation was as efficient as the i.c. route of inoculation. Further studies were not done by this route because of the rapidity of dissemination of virus in the brain and the cumbersome anatomical barriers that restrict the study of virus transit to the brain. A more simple paradigm was obtained by using inoculation in the footpad, as described below.

Footpad route. The 12 animals inoculated with stock virus by this route developed disease 45 to 60 days later. We investigated the time required for virus to enter the sciatic nerve from the site of inoculation by sectioning the nerve on days 1, 2, 3, and 5 after inoculation in the footpad. Two to five animals were used at each time. Disease was prevented only by sectioning the nerve within 1 day after inoculation. Section of the nerve after this time did not prevent development of disease. This demonstrated that the infectious agent entered the nerve within 2 days after inoculation into the footpad.

Examination of tissues from the sick animals showed a pattern of viral infectivity, viral antigen, and inflammation in the brain, spinal cord, dorsal root ganglia, and sciatic nerve that was indistinguishable from that resulting after i.c. inoculation. To determine whether infection in the brain via the footpad inoculation occurred by transport of the agent in the sciatic nerve or by other means of transport to the brain, we sectioned the sciatic nerves of 13 rats and inoculated the animals in the footpad of the same foot 2 days later. Only 1 of 13 animals inoculated in this experiment developed disease after an observation period of 112 days, and none of the clinically well rats developed antibodies to BDV antigen. Four of these rats were then inoculated in the footpad of the contralateral hind leg with an intact sciatic nerve. All four animals developed acute disease by 60 to 70 days after this

TABLE 4. BDV titers after footpad inoculation

Days after footpad inoculation	Titer ($TCID_{50}$ per g of tissue sample) in:				
	Sciatic nerve	Dorsal root ganglia	Spinal cord		Brain
			Lumbar	Cervical	
7	0	0	0	0	0
14	10^3	10^3	10^5	10^5	10^5
21	$>10^3$	10^3	10^5	10^6	10^7
56	10^2	10^3	10^4	10^7	10^7

inoculation. This experiment established the role of neural transport of the agent to the brain.

Having established that the footpad-sciatic nerve was a reliable conduit of BDV to the brain and that animals developed disease predictably after this inoculation, we inoculated a number of rats via this route and examined tissues from individual animals killed at sequential intervals after inoculation. The purposes of this experiment were first to determine the course of virus dissemination from the site of inoculation to the brain, second to identify the sites of virus replication in various neuronal groups along this route, and third to correlate these with the onset of disease.

Groups of three animals examined at 7, 9, and 11 days after inoculation had neither infectivity nor antigen in brain, spinal cord, dorsal root ganglia, or sciatic nerve. Beginning on day 14, two rats were killed at weekly intervals and homologous samples of tissues from brain, cervical spinal cord, lumbar spinal cord, dorsal root ganglia, and sciatic nerves were pooled and assayed for infectivity. The highest titers were found in homogenates of brain (Table 4). Other rats were perfused and examined for viral antigen and pathological changes. Tissues from rats examined before day 35 had no antigen or pathological changes. One of two animals killed on day 35 and both killed on day 42 had viral antigen in several neurons in the dorsal root ganglia but in none of the other tissues. In the latter two animals antigen was confined to the dorsal root ganglia on the side ipsilateral to the inoculation site. One animal had extensive infiltration of mononuclear inflammatory cells in the ganglia and sciatic nerve. Several fibers in the sciatic nerve and dorsal columns had undergone Wallerian degeneration. No inflammatory changes were seen in any other nerve tissues. This rat was clinically normal at the time it was killed. Two rats were killed for morphological studies on day 60. One animal had developed acute clinical disease 5 days before perfusion. Examination of tissues from the clinically normal rat showed viral antigen in the spinal ganglia, in neurons in the medulla oblongata, and in dense concentration in nerve fibers in this area of the hind brain. In addition, viral antigen was found in

FIG. 3. Rats were injected i.c. with BDV and perfused with 4% paraformaldehyde, and tissue samples were mounted in paraffin. Slides were processed for BDV-specific antigens with the avidin-biotin system with postinfection sera as a source of anti-BDV antibody. (A) This section of dorsal root ganglion was counterstained with hematoxylin to demonstrate the influx of mononuclear inflammatory cells (small arrow). Large dorsal root ganglion cells containing BDV antigen are indicated (long arrows). For reference a normal cell is shown (open arrow). (B) The hippocampal neurons of the CA3 region containing BDV antigens (arrows). No hematoxylin counterstain was used for this or any of the tissues in the following panels because the stained antigen was adequate to outline the nerve cell bodies and their processes. Infection of this area, with associated mononuclear inflammation, heralded the onset of clinical disease. (C) This section of spinal cord shows marked antigen deposition in the grey matter, in contrast to the white matter (small arrow). Ventral horn cells which control motor functions are seen to contain antigen in their nuclei and cytoplasm (large arrows). (D) BDV-specific antigens were seen within nerve fibers of the sciatic nerve (arrows). (E) A collection of neurons in the medulla containing BDV-specific antigen (long arrow). Fiber tracts that do not contain the antigen (short arrows) pass near areas of cell processes carrying BDV antigen (block arrow). (F) Pyramidal cells in the cortex outlined by BDV-specific antigen which fills the perikaryon (block arrow) as well as the cell processes (small arrow).

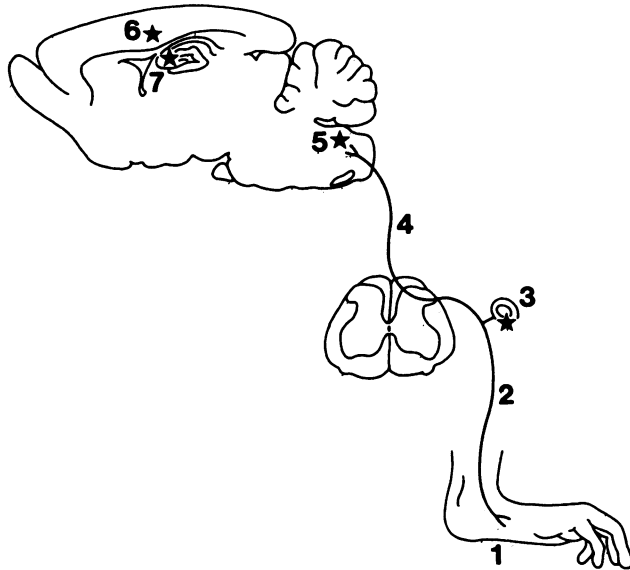

FIG. 4. Delineation of anatomic pathway of BDV transit from the foot to the brain. Virus deposited in the foot (site 1) was transported in retrograde fashion along processes of sciatic nerve (site 2) to the first of several sites of replication (★), neurons in the dorsal root ganglion (site 3). Infectious progeny was transmitted in the cell processes located in the dorsal columns of the spinal cord, shown in cross section (site 4), and spread transsynaptically to neurons in the medulla of the brain, shown in sagittal section (site 5). Virus continued to spread in the brain to the cortex, in which viral antigen was found next in pyramidal cell neurons (site 6). Finally, virus spread from these to the hippocampal neurons (site 7). A mononuclear cell inflammatory response ensued, and the animal developed acute clinical signs of disease. Sectioning of the sciatic nerve (site 2) before virus inoculation in the foot prevented infection and disease.

the large pyramidal neurons in layers 4 and 5 of the frontal cortex. Hippocampal neurons were unaffected. Examination of the sick rat showed distribution of viral antigen similar to that in rats inoculated i.c. This included dense concentrations of antigen in the CA3 and CA4 regions of the hippocampus and severe encephalitis. Infection (expression of viral antigen) in hippocampal neurons was therefore thought to be a major factor in triggering signs of disease. Examination of tissues from rats with more severe disease showed viral antigen in widely diverse groups of neurons, such as that seen typically after i.c. or i.n. inoculation. This wide dissemination of virus occurred after infection became established in the hippocampus.

Intramuscular route. To investigate whether entry of the virus into the nerve required prior replication in the muscle, 30 12-week-old male Lewis rats were injected with 10^5 $TCID_{50}$ of BDV in the right gastrocnemius muscle of the hind limb. Four animals were killed at days 0, 1, 2, 3, 6, and 31, and the gastrocnemius muscles were examined for viral antigen and infectivity. Six animals were observed for onset of disease. All of these rats became ill with signs of Borna disease between days 32 and 38 after inoculation. On day 0 (1 h after inoculation) 10^3 $TCID_{50}$ of BDV was recovered from the muscle; the virus recovered declined to 10^2 by day 1 and was no longer detectable by 2, 3, 6, and 31 days after inoculation. No viral antigen was detectable at any time in the gastrocnemius muscle. No evidence of myositis was seen in any of these tissue samples.

DISCUSSION

These studies showed that the pathogenesis of the neurological disease caused by BDV in rats was determined by the tropism of the agent for neural cells and the failure of antibodies to limit spread of the virus during the incubation period of the disease. The disease resulted only after virus had replicated in neurons of the hippocampus. Our results suggest that the incubation period of the disease was the time required for the agent to be transported from endings of peripheral nerves at the point of inoculation to the hippocampus via dendritic-axonal processes. The agent was amplified by replication in the soma of interconnecting neurons and eventually reached the hippocampal neurons, presumably by spreading across synaptic connections. Provided the agent overcame natural physiological barriers to enter the nervous system from the peripheral sites, it became free to replicate in neurons and circulate indefinitely in the dendritic-axonal network of the nervous system. The change in intracellular localization of antigen from massive accumulation throughout the cell bodies of certain neurons during acute disease to slight accumulation in cell nuclei during chronic disease seemed correlate with loss of the former cells from the brain. This may have been due to immunopathologic consequences of infection (16).

The inefficiency of the i.v. route of inoculation and the success of the footpad route in providing access of the virus to the brain highlighted the highly unusual biological properties of this agent. Whereas transport of viruses across vascular endothelium from blood vessels to the brain is the route of invasion of brain by many neurotropic viruses (9), multiple i.v. injections of stock BDV were ineffective in causing infection in most of the inoculated rats. Furthermore, these inoculations did not elicit neutralizing antibodies. Failure to cause infection may have been due to rapid clearance of the agent from the blood after i.v. inoculation and to inability of the virus to replicate in vascular endothelial cells or in fixed mononuclear tissue phagocytes which remove it from the circulating blood. The antibody response of some of the i.v.-inoculated animals may have been the result of antigenic stimulation by the large amount of viral antigen in the infectious brain homogenate. Although the viral inoculum could be recovered in diminishing titer up to 24 h from intramuscular sites of inoculation, no viral antigen, inflammation, or increasing viral titers were detected. Local replication of virus is not required for dissemination and infection of the CNS.

Earlier studies on dissemination of BDV in the nervous system had clearly established the importance of transport of the agent via nerve processes. The elegant experiments of Krey et al. (13) showed that laser cauterization of the optic disk could prevent spread of virus from the eye to the brain. This concept was expanded further by the illustration that the virus could invade the brain almost as efficiently after inoculation i.n. as after direct i.c. inoculation (S. Hertzog and R. Rott, unpublished data). The i.n. route is a well-established pathway to the brain because dendritic processes of olfactory receptor neurons (olfactory rods) end beyond the free surface of the nasal mucosa. These nerve endings thus provide a direct conduit of virus to the brain (9, 21).

The ability of the virus to reach the brain after inoculation into the footpad, coupled with the failure of neurectomized rats to become ill after inoculation into the ipsilateral foot (with a transected sciatic nerve), further established the importance of the nerve conduit (Fig. 4). Induction of disease in these animals at a later period after a second

inoculation of virus into the contralateral foot (with an intact sciatic nerve) reinforced the concept of the neural route as the pathway for virus invasion of the brain. Since the incubation period of disease after the second inoculation was similar to that in the virus control group and since two animals which did not receive the second inoculation remained clinically healthy after 150 days, we concluded that it was virus in the second inoculum that caused disease. BDV lends itself to experimental manipulation of this kind because of its strict requirement for replication in neurons and its failure to induce protective immunity.

Reconstruction of the events after footpad inoculation suggested that the virus did not replicate at any site in the animal until it had been transported by sensory nerve fibers (Fig. 4, site 2) to neurons in the dorsal root ganglia (site 3). The agent replicated in these neurons and was then transported in axons of these cells through the dorsal columns of the spinal cord to their synaptic terminals in the gracilis nucleus in the medulla (site 5) and also distally, down the fiber tracts in the sciatic nerve (site 2). Infectivity without antigen had been observed in the brains of rats 14 days after inoculation in the footpad; it is possible that infectious virus detected in the brain at that time was produced in neurons of the dorsal root ganglia and had spread rostrally in the cell processes. Since the processes of the neurons in the dorsal root ganglia (L3 and L5) begin in the feet of the animal and terminate in the medulla, replication of the virus in only this group of neurons may have been enough to facilitate the spread of virus from the foot to the brain with a single amplification step. We speculate that the dense concentration of antigen in fiber tracts in the medulla represented a concentration of viral antigen and infectious particles approaching these synaptic terminals. Presumably the agent traversed these synaptic junctions because of the later appearance of viral antigen in neurons in the brain.

A similar transneuronal transport mechanism is invoked to explain the appearance of viral antigen later in the pyramidal cells of the cerebral cortex. Since antigen appeared in the hippocampus after antigen was detected in the cortex, the agent must have been transported either along fiber tracts connecting these groups of cells or via more circuitous neuronal pathways. Replication in the hippocampal neurons then provided the signal for inflammation and disease. Virus dissemination from the hippocampus to other neurons in the central, peripheral, and autonomic nervous systems was very rapid. This dissemination was similar, irrespective of the route of inoculation. Infection in the hypothalamus could explain eating disorders and obesity (3), and the infection in motor neurons of the brain stem and spinal cord as well as cells in the deep cerebellar neurons may result in ataxia.

In summary, the pathogenesis of BDV infection differs from that caused by most viruses because of the strict neurotropism of the virus and the lack of a role for antibodies in preventing or limiting virus replication. The highly neurotropic nature of the agent is reminiscent of that of rabies virus (9). In another analogy, the lack of control of the agent by an immune response together with the i.c. dose-response curve (dose-dependent variability in incubation period followed by classic clinical disease) is reminiscent of host interaction with the scrapie prion agent (11). Although infection in nonneural tissues is an integral part of the pathogenesis-ecology of both of these viruses (the salivary gland in rabies and the spleen in scrapie), a nonneural site of replication has not been identified for BDV. It is not known whether rodents are important in nature for maintaining BDV and, if so, whether the pathogenesis of infection in these animals bears any relationship to the infection in laboratory rats. However, this model of BDV infection in Lewis rats provides insight into a remarkable pathogenesis that is unparalleled by any known infectious agent.

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