Barrier-bred cats were inoculated intracerebrally with either the rabbit-adapted Borna disease virus (BDV) strain V or a newly isolated feline BDV, obtained from a cat with natural staggering disease (SD). Three out of eight inoculated cats developed neurological signs and non-suppurative encephalitis; all three recovered from the acute stage of disease. Sero-conversion and the development of neutralizing antibodies occurred in all of the virus-inoculated cats. In addition, cats inoculated with feline BDV showed an early peripheral T cell response not present in cats inoculated with BDV strain V, suggesting that the feline virus exerted a more vigorous effect on the immune system. Using immunohistochemistry and a reverse transcriptase-polymerase chain reaction assay, BDV-specific antigen and nucleic acid could be demonstrated in brain samples from each cat with encephalitis, showing that incomplete viral clearance was probably responsible for the maintenance of inflammation. The successful induction of neurological signs and encephalitis in one cat infected with feline BDV, together with the detection of BDV-specific antigen and nucleic acid in the brain, provides strong evidence for the notion that BDV is the etiological agent behind feline SD.

Key words Borna disease virus · Cat diseases · Encephalomyelitis · Immunopathology
acute viral infection such as feline panleukopenia than
with BD.

To investigate further the susceptibility of cats to BDV
infection, we performed an experimental transmission
study, using barrier-bred, specific pathogen-free (SPF)
cats. Two different strains of virus were used: the labora-
tory reference strain V and the recently isolated feline
BDV. Following intracerebral inoculation, the animals
were closely observed and immunologically monitored
for 6 months. We report on the appearance of neurological
signs and encephalitis in some of the inoculated cats. All
animals survived the infection, pointing to the importance
of an early cellular as well as humoral immune response
for viral clearance and recovery from disease.

Materials and methods

Animals

Twelve barrier-bred 5-month-old health-monitored cats of the strain
ICo:Fec Eur, Tif, obtained from IFFA-CREDO (L’Arbresle, France),
were used for the study (six male, six female). All cats were vacci-
nated against feline panleukopenia, feline herpes and calici virus
infection and rabies (Leucorifelin and Quadratic, Rhone-Merieux)
at 8 weeks of age, receiving booster injections at 13 weeks of age.
They were serologically negative for feline leukemia virus, feline
immunodeficiency virus and feline coronavirus (Virus Laboratory,
National Veterinary Institute, Sweden). Before the start of the ex-
periment, the cats were conditioned for 6 weeks, divided into three
separate groups (I–III) of two males and two females each. During
this period, all males were neutered and blood samples were taken
for flow cytometry analysis and white blood cell (WBC) counts.
Groups I and II were kept freely roaming in rooms with controlled
light cycle (12 h light/12 h dark) and temperature (21 ± 1°C). Each
animal received an intravenous injection with cyclophosphamide (Cyklo-
 fosfamide, Orion), diluted in sterile water to a concentration of 20 mg/
ml, at a dose of 10 mg/kg body weight (Table 1). Cats which were
not treated with cyclophosphamide were killed in the same manner on day 183 p.i.

Preparation of virus suspensions

Two different virus suspensions were used for inoculations:
1. BDV strain V [19], a rabbit-adapted laboratory strain originally
isolated from horses. Infected rabbit brain suspension was diluted
1:10 in phosphate-buffered saline (PBS) and stored in aliquots of 1
ml at −70°C before use. The titer of the suspension was 7 × 10^6 fo-
cus forming units (ffu)/ml.
2. Feline BDV, originally obtained from the brain of a cat with SD
and thereafter passaged twice intracerebrally in newborn Wistar
rats [24]. Infected rat brain suspension was diluted 1:10 in PBS
and stored in aliquots of 1 ml at −70°C before use. The titer of the
suspension was 2 × 10^6 ffu/ml.

Experimental design

On day 0, each cat was anesthetized using a mixture of medetomi-
dine (Domitor, Lääkefarmos/Farmos) at a dose of 0.1 mg/kg intra-
muscularly (i.m.) and ketamine (Ketalar, Parke-Davis) at a dose of
5 mg/kg i.m. Serum was obtained and stored at −20°C for BDV an-
tibody determination. Four cats (group I) were inoculated with
BDV strain V and four cats (group II) with the feline BDV isolate
(Table 1). All inoculations were given intracerebrally (0.3 ml/cat).
A surgical site was prepared aseptically over the left parietal cor-
tex, the skin was incised with a scalpel blade and a small hole
drilled through the skull using a surgical dentist’s drill. The virus
suspensions were introduced into the left parietal cortex using a
0.6x25-mm needle. Of the cats in group III, two were given a nor-
al rabbit brain suspension intracerebrally, while the remaining
two were left as uninoculated controls.

The cats were observed twice daily, for at least 1 h each time,
for signs of illness and/or changes in behavior. On days 14, 25, 35,
63, 91 and 119 all cats were anesthetized as described and serum,
EDTA blood samples and heparinized blood samples were ob-
tained for serology, blood chemistry, hematology and flow cytom-
etry. Cerebrospinal fluid (CSF) was collected by cisternal puncture
and analyzed immediately for cell content.

On day 154 post inoculation (p.i.), two cats from each group re-
ceived an intravenous injection with cyclophosphamide (Cyclofos-
hamide, Orion), diluted in sterile water to a concentration of 20 mg/
ml, at a dose of 10 mg/kg body weight (Table 1). Cats which were
not treated with cyclophosphamide were killed by pentobarbital
overdose given intraperitoneally on day 154 p.i. The cyclophos-
phamide-treated cats were killed in the same manner on day 183 p.i.

Final blood samples and CSF were taken from all cats, and
postmortem examinations were performed within 1 h of death.

Tissue processing

The brain and spinal cord were removed intact from all cats at
carcopsy. The brain was divided into two halves by a longitudinal
cut through the midline of the corpus callosum and the brain stem.
From the right half, small pieces (approximately 5x3 mm) were
taken from different regions, snap frozen in liquid nitrogen and

Table 1 Summary of experimental groups (M male, F fe-
nale, BDV Borna disease virus, BDV V BDV strain V, i.c. intracerebrally, i.v. intrave-
necously, susp. suspension)

| Group | Cat/Sex | Schedule of infection |
|-------|---------|-----------------------|
| I     | 1/M     | BDV V, 0.3 ml i.c., day 0 |
|       | 2/F     | BDV V, 0.3 ml i.c., day 0 |
|       | 3/M     | BDV V, 0.3 ml i.c., day 0. Cyclophosphamide 10 mg/kg i.v., day 154 |
|       | 4/F     | BDV V, 0.3 ml i.c., day 0. Cyclophosphamide 10 mg/kg i.v., day 154 |
| II    | 5/F     | Feline BDV, 0.3 ml i.c., day 0 |
|       | 6/M     | Feline BDV, 0.3 ml i.c., day 0 |
|       | 7/F     | Feline BDV, 0.3 ml i.c., day 0. Cyclophosphamide 10 mg/kg i.v., day 155 |
|       | 8/M     | Feline BDV, 0.3 ml i.c., day 0. Cyclophosphamide 10 mg/kg i.v., day 155 |
| III   | 9/F     | Uninoculated control |
|       | 10/F    | Uninoculated control. Cyclophosphamide 10 mg/kg i.v., day 154 |
|       | 11/M    | Normal rabbit brain susp., 0.3 ml i.c., day 0 |
|       | 12/M    | Normal rabbit brain susp., 0.3 ml i.c., day 0. Cyclophosphamide 10 mg/kg i.v., day 154 |
stored at −70°C for later determination of viral RNA by reverse transcriptase (RT)-polymerase chain reaction (PCR). The left half was fixed in buffered 10% formalin and cut in serial coronal slices including the olfactory bulb, frontal cortex, basal ganglia, parietal cortex, thalamus, hippocampus, temporal and occipital cortex, mesencephalon, caudal colliculus, pons, lateral cerebellar hemisphere and the medulla oblongata. After embedding in paraffin, 4-μm-thick sections were cut and stained with hematoxylin and eosin (H&E) for light microscopy. Specimens from the cervical, thoracic and lumbar segments of the spinal cord, as well as from the cranial mesenteric ganglion and the sciatic nerve, were likewise processed.

Extraneural tissue specimens were sampled from the eyes, retropharyngeal lymph nodes, spleen, liver, kidneys, heart, lung, mandibular gland, pancreas, jejunum, adrenal gland, thymus, uterus, ovaries and the sartorial muscle. Samples from all these tissues were formalin fixed and processed for histopathology; in addition, some samples were snap frozen in liquid nitrogen and stored at −70°C.

Immunohistochemistry

For detection of BDV antigen, two different rabbit BDV-specific antisera (LL2, BP11) and a monoclonal antibody (mAb) Kha2, recognizing the 24-kDa protein of BDV [19] were used on paraffin sections of the brain. The avidin-biotin immunoperoxidase complex (ABC) method [14] was used with 3-amino-9-ethylcarbazole (AEC) as the chromogen, as described previously [23].

Two cats from groups I and III and three cats from group II were examined immunohistochemically. The parietal cortex and hippocampus were selected for examination. For the three cats with encephalitis (see Results), all brain regions with inflammatory changes were evaluated immunohistochemically.

RNA extraction

Total RNA was extracted from tissue samples using the TRizol reagent (Life Technologies). The RNA was dissolved in nuclease-free water and stored at −70°C. RNA concentration was determined by spectrophotometry at 260 nm. RNA was prepared from the following brain regions of all cats: basal ganglia, hippocampus, cerebral cortex and mesencephalon. From the three cats with encephalitis (see Results), all brain regions with inflammatory changes were evaluated immunohistochemically.

Primers, probe and reagents

The oligonucleotides were designed according to published sequences of the BDV genome encoding the 38/40-kDa protein [7], using the computer program OLIigo version 4.0. The primer sequences have been described previously [38]. For Southern hybridization, the following 32P-labeled oligonucleotide located between the inner primers was used: 5′-AAAAGCCAGGGCCGAGCAGAT-3′. The oligonucleotide probe was synthesized in a Gene Assembler Plus (Pharmacia LKB). The M-MLV RT and the corresponding buffer system were obtained from Life Technologies. All PCR reagents including nucleotides, 10× reaction buffer and Taq polymerase (AmpliTaq) were obtained from Perkin-Elmer Cetus.

RT-PCR assay and nucleic acid hybridization

Prior to PCR amplification, cDNA was synthesized by the RT reaction as described previously [38]. The external primers were used to prime selectively either the positive-strand viral mRNA or the negative-strand viral genomic RNA. PCR amplifications were carried out in 50-μl volumes containing 5 μl of the RT reaction. Reaction solutions comprised 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200 μM each dNTP, 1 μM of each primer and 0.5 U Taq polymerase. The samples were processed through 30 cycles of 1 min at 94°C, 1 min at 54°C and 1.5 min at 72°C. Following the first round of PCR, a second (nested) round was performed using 2 μl of the first reaction with the internal primer pair for 30 cycles of 1 min at 94°C, 1 min at 52°C and 1.5 min at 72°C.

One fifth (10 μl) of each reaction after the nested PCR was analyzed by electrophoresis in a 1% agarose gel in the presence of 2 μg/ml ethidium bromide. The oligonucleotide probe was labeled with [γ-32P]ATP (sp. act. 5000 Ci/mmol, Amersham) using a T4 nucleotide kinase (Pharmacia). Southern blotting and hybridization were carried out using standard methods [33]. To verify the integrity of the RNA preparation, the amplification of a 248-bp β-actin mRNA served as an internal control [38].

Hematology and clinical chemistry

Hemoglobin concentration, WBC counts, differential cell counts and CSF cell counts were determined according to standard procedures at the Department of Clinical Chemistry, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences. The absolute numbers of neutrophils and lymphocytes were calculated from the total leukocyte and differential counts. Correction for contamination by peripheral blood in the CSF was made by subtracting the number of 1 WBC for every 100 erythrocyte/ml [32]. Total protein concentration in serum was determined by the Biuret method and serum protein electrophoresis was performed using a Paragon system (Beckman instruments).

Preparation and staining of cells for flow cytometry

Heparinized blood (3 ml) was diluted 1:1 with RPMI 1640 (Gibco) and layered upon 7 ml Ficoll-Paque (Pharmacia). Tubes were centrifuged at 600 g for 30 min, peripheral blood mononuclear cells were isolated from the interface and the concentration of cells was determined by counting in a Bürker chamber. The cells were transferred to polypropylene tubes, 5×10^6 cells/tube, and washed once in RPMI containing 10% fetal calf serum (FCS). For identification of lymphocyte subpopulations, mAbs against feline CD4 (CAT30A, VMRD), CD8 (ICD8, Southern Biotechnology), CD3-like antigen/pan T (CF54A, VMRD) and CD21-like antigen/B subset (F46A, VMRD) were used. A mAb against bromodeoxyuridine (M744, Dakopatts) served as isotype control. The cells were incubated with the primary antibodies (all diluted to a final concentration of 20 μg/ml) for 30 min on ice, thereafter washed once with RPMI-FCS and incubated with a fluorescein isothiocyanate (FITC)-conjugated F(ab')2 rabbit anti-mouse IgG (F313, Dakopatts) for 30 min in the dark on ice. The cells were washed once with RPMI-FCS and resuspended in PBS containing 1 μg/ml propidium iodide (PI, Sigma).

Flow cytometry, computer analysis and statistical methods

Flow cytometry was performed using a FACStar PLUS flow cytometer (Becton Dickinson Immunocytometry Systems) with standard optical equipment. Cells were illuminated with an argon-ion laser (Spectra-Physics 2016) tuned to 488 nm and operating at 200 mW. Data were acquired using the FACStar PLUS software version 2.01. Off-line analysis was performed using the LYSYS version 1.62. Data from 20000 cells were acquired. The following parameters were collected: forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1) and PI fluorescence (FL3). Dead cells were identified by their uptake of PI, and were excluded from further analysis. The lymphocytes were identified by their size (FSC) and granularity (SSC). The FITC fluorescence of the lymphocytes was further evaluated. The discrimination between positive and negative cells was set by placing the discrimination so that 2% positive cells were detected in the sample stained with isotype control for each cat. This number was then subtracted from the frequency of positive cells in the samples stained with antibodies to feline leukocyte surface antigens.
Statistical analysis of differences between the control group and the two exposed groups was performed with the Mann-Whitney U-test. The frequencies of cells positive for each of the markers CD4, CD8, CD3-like/pan T and CD21-like/B-subset in samples from the two infected groups were compared with frequencies of the control group at each sampling point.

Indirect immunofluorescence test

For the detection of BDV-specific antibodies in serum, an indirect immunofluorescence test (IFT) was performed as described previously [1], using a double-stain technique. In brief, young rabbit brain cells infected with BDV and seeded on coverslips were incubated with equal volumes of cat serum and the BDV-specific mAb Kfu3, recognizing the 38/40-kDa protein of BDV [19], followed by a second incubation with FITC-labeled goat anti-cat IgG (Dianova) and tetramethyl rhodamine isothiocyanate-labeled (F(ab')2) rat anti-mouse IgG (Dianova). Cat sera were ranked as containing specific antibodies only if they induced a pattern of fluorescence identical to that induced by Kfu3. All positive samples were endpoint titrated.

Neutralization test

For evaluation of the neutralizing capacity, cat sera were decomplemented, diluted and subjected to a neutralization test based on the principle of plaque reduction, as described previously [19]. Sera from BDV-infected, as well as uninfected, rabbits were used as positive and negative controls. Titers were expressed as the dilution at which the number of foci was reduced by 50%.

Results

Clinical signs and clinical pathology

In group I (inoculated with BDV strain V), one cat (no. 1) showed bilateral protrusion of the third eyelid 15 days p.i. The cat was also mewing more than usual. On day 20 p.i., cat 1 was shy and reserved. During the following 2 days, a clear personality change was observed. The cat was extremely shy and cautious, ran away when approached and assumed a peculiar staring gaze. Between days 24 and 27 p.i., clinical signs aggravated. The cat was moving in circles, especially when approached, and seemed unable to jump up and down normally. A slight to moderate hind leg ataxia was observed. After day 27, movements as well as behavior were normalized; however, for some days the cat seemed to be hypersensitive to sound and touch and had small tremors in the skin, most apparent in the eyelids and whiskers. An altered voice was also noted. Between days 30 and 154 p.i., there were no changes in the clinical condition of this cat. The altered voice remained to the end, as did a certain mental dullness. Another cat in group I (no. 4) showed a slightly stiff gait and ataxia in the hind legs 2.5 months p.i. These signs remained constant until euthanasia.

In group II (inoculated with feline BDV), one cat (no. 5) showed signs of rapid breathing and disturbed proprioception in the front legs on day 1 p.i.. These symptoms disappeared completely on day 2 and were interpreted as due to traumatic injury from the inoculation procedure. Between days 11 and 16, another cat in this group (no. 7) showed bilateral protrusion of the third eyelid. At 2.5 months p.i., a third cat (no. 6) was observed to hold its neck in a stiff position, sometimes turning the head with a twitchy movement, and also showing signs of slight hind leg ataxia. These clinical signs remained until the cat was killed on day 154 p.i.

Hemoglobin concentration remained within normal limits (80–150 g/l) throughout the study. A transient decrease in WBC was observed in groups I and II 14 days p.i. True leukopenia (3.0 × 10⁹–5.0 × 10⁹ cells/l) occurred in three cats (group I: cats 1, 3; group II: cat 7), due mainly to neu-

| Cat  | 1a | 1b | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  |
|------|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|--|
| 1    | –  | ac | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ax  |
|      | +  | +  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +  |
|      | +  | mg | mg  | mg  | mg  | mg  | mg  | mg  | ax  | ax  | mg  | mg  | ax  | ax  | mg  | ax  | ax  | +  |
|      | +  | +  | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | +  |
|      | +  | +  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +  |
|      | +  | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | nph | +  |
| 4    | –  | ac | ac  | ac  | ac  | ac  | mg  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ax  |
|      | +  | +  | +   | +   | +   | +   | ax  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +  |
|      | +  | +  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +  |
| 6    | –  | –  | –   | –   | –   | –   | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | ac  | +   |
|      | +  | +  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +  |
tropenia. At 25 days p.i., WBC counts were increasing in both groups. A minor decrease was noted in group I 35 days p.i. After this, WBC counts were stabilized. Total protein concentration and electrophoretic patterns of serum proteins remained within normal values in all three groups.

In group I, a moderate increase in CSF leukocytes was observed in two cats (nos. 3, 4) 14 days p.i. At the same time two cats in group II (nos. 6, 7) also showed a moderate increase in CSF leukocytes. In both groups, mononuclear cells predominated. The highest CSF cell count was detected in cat 4 (42 WBC/µl vs normal < 5 WBC/µl). On day 25 p.i., cats 1, 4 and 6 showed signs of pain and increased extensor tone of the front legs during cisternal puncture. Because of this, CSF sampling was interrupted and no CSF analysis could be performed. Throughout the remainder of the study, CSF leukocyte counts were normal in both of the inoculated groups.

**Fig. 1** Diagrammatic representation of brain regions examined (see Table 2). Modified after Luttrell and Bang [25]

**Fig. 2A–D** Histopathological and immunohistochemical findings in cats experimentally infected with Borna disease virus (BDV). A Adventitial mononuclear cell cuffs in the basal ganglia of cat 1, inoculated with BDV strain V. B Axonal degeneration and loss of myelin in the cervical spinal cord of cat 4, inoculated with BDV strain V. C Plasma cells in close association with neurons in the frontal cortex of cat 2, inoculated with BDV strain V. D Pons of cat 4, inoculated with BDV strain V. Immunostaining with a polyclonal antibody against BDV. A–C Hematoxylin and eosin, D avidin-biotin-peroxidase complex method; A, B, D × 168, C × 672
The control cats in group III did not develop any clinical signs during an observation period of 6 months. Their hemoglobin concentrations, WBC counts, serum protein concentrations and CSF cell contents remained within normal limits throughout the study.

Histopathology

Histopathological examination revealed encephalitis in the three cats with neurological signs. In group I, cat 1 had a severe non-suppurative meningoencephalitis extending from the frontal cortex through the basal ganglia and the brain stem (Table 2; Figs. 1, 2A). The lesions were characterized by broad adventitial cuffs consisting of mononuclear cells (lymphoid cells, cells resembling macrophages, and plasma cells), presence of inflammatory cell infiltrates in the neural parenchyma, occasional neuronophagia, microgliosis and scattered axonal degeneration. In the cerebral cortex and hippocampus, plasma cells were occasionally observed close to neurons. Inflammatory changes were more pronounced in the white matter than in the gray matter, particularly in the internal capsule at the level of the basal ganglia and in the periventricular white matter of the rostral brain stem. In the spinal cord, ventrolateral axonal degeneration and loss of myelin were observed at all levels.

Another cat in group I, cat 4, showed histopathological evidence of a moderate non-suppurative encephalitis (Table 2), with inflammatory lesions almost completely confined to the subcortical white matter. Adventitial infiltrates were less extensive than in cat 1, and there were no signs of neuronophagia. Occasional plasma cells appeared adjacent to hippocampal neurons. In the spinal cord, ventrolateral axonal degeneration and loss of myelin occurred at all levels (Fig. 2B).

The only inflammatory change observed in the CNS of the other cats in group I (nos. 2, 3) was the presence of scattered plasma cells close to neurons and glial cells of the cerebral cortex and hippocampus (Fig. 2C). In addition, cat 2 showed a slight mononuclear cell infiltration in the choroid plexus of the third ventricle and cat 3 had a single, thin adventitial mononuclear cuff in the internal capsule at the level of the basal ganglia.

Cat 6 of group II had a mild non-suppurative encephalitis localized in the laterobasal temporal cortex (Table 2). There were scattered adventitial cuffs consisting of lymphoid cells and a few plasma cells both in the gray matter and at the border between gray and white matter. Occasional plasma cells were found in the vicinity of neurons in the frontal, parietal and temporal cortex. The other cats (nos. 5, 7, 8) in group II lacked encephalitic lesions. Cat 7 had a minimal adventitial infiltrate in the internal capsule at the level of the basal ganglia. Cat 5 had a slight mononuclear infiltrate in the choroid plexus of the third ventricle. Scattered plasma cells in the vicinity of morphologically normal neurons were observed in the cerebral cortex of all three cats.

With a few exceptions, extraneural tissues were histopathologically normal. Cat 4 in group I had markedly enlarged retropharyngeal lymph nodes with follicular hyperplasia. Three cats in group I and two in group II had larger and more active germinal centers in the spleen compared to group III control cats. Cat 5 in group II had a slight interstitial infiltrate consisting of lymphoid cells in the cortex of the kidney.

The control cats in group III showed no histopathological abnormalities of the CNS or any of the examined extraneural tissues.

Detection of BDV antigen and nucleic acid

In cat 1 (inoculated with BDV strain V), BDV antigen was found within neurons and glial cells in all examined brain regions except the olfactory bulb and the hippocampus. Positively stained cells were scattered in the cerebral cortex, often in regions adjacent to inflammation in the underlying white matter. Usually, the antigen-containing cells appeared only in the cortex of the different regions, being notably absent from the basal and thalamic nuclei as well as from the immediate vicinity of adventitial cuffs. The staining of neurons was predominantly cytoplasmic and tended to be somewhat granular. The majority of the antigen-containing neurons had a shrunken appearance. In cat 4 (inoculated with BDV strain V), a few antigen-contain-
Fig. 4 Flow cytometric investigation of lymphocyte subsets in peripheral blood of control cats (a, d), cats inoculated with BDV strain V (b, e) and cats inoculated with feline BDV (c, f). a–c Changes in the frequencies of pan T-positive cells (●) and B(sub)-positive cells. d–f Changes in the frequencies of CD4-positive cells (□) and CD8-positive cells (■). * denotes a significant elevation ($P < 0.05$) and † a significant decrease ($P < 0.05$) when values were tested against the control group (Mann-Whitney U-test). Bars represent standard errors of the mean.
ing neurons and glial cells were found in the deep nuclei of the cerebellum, and in the cerebral cortex. The positively stained neurons did not show any cytopathic changes (Fig. 2D).

Of the cats in group II (cats inoculated with feline BDV), cat 6 had a few antigen-containing neurons and glial cells in the laterobasal temporal cortex, close to a region with several adventitial cuffs. BDV antigen could not be detected immunohistochemically in any of the other group II cats.

Following selective priming for viral mRNA or viral genomic RNA in the RT reaction, no amplification products were detected by agarose gel electrophoresis after the first PCR. After a second (nested) round of PCR with the internal primer pair, the expected 444-bp BDV-specific band could be visualized in samples from at least one brain region in all cats with histopathological evidence of encephalitis. The specificity of the PCR products was verified by Southern blotting and hybridization of a 32P-labeled oligonucleotide probe to the amplified DNA (Fig. 3).

Cat 1, which had the most severe inflammatory lesions, was positive for BDV mRNA in the cerebral cortex and thalamus, while the hippocampus of this cat only contained viral genomic RNA. In cat 4, BDV mRNA could be demonstrated in the cerebral cortex and the cerebellum. Cat 6 was positive for BDV mRNA in the hippo-
Peripheral cellular and humoral immune response

Cats in group I showed an increase in the frequency of pan-T-positive lymphocytes 25 days p.i. (Fig. 4b). This increase, however, was not significant. At 35 days p.i., there was a significant increase of B cells and a decrease of T cells. After day 35, the population of T cells in the peripheral blood approached control values. A decrease in the frequency of CD8-positive cells was consistent throughout the study, and was significant at 35, 63, 91 and 154 days p.i. (Fig. 4e).

In group II, there was a significant increase in the frequency of pan-T-positive lymphocytes 14 days p.i. (Fig. 4c). The CD4- and CD8-positive subpopulations increased simultaneously, with a slight predominance of CD4-positive T cells (Fig. 4f). The frequencies of T cells then declined, reaching a minimum 35 days p.i. At the same time, a significant increase in the frequency of B cells was observed. This switch in T and B cell frequencies was more pronounced in group II than in group I. During the remainder of the study, the frequencies of T cells increased and approached control values. However, a significant decrease in CD4-positive cells was observed at 63 and 154 days p.i., and a significant decrease in CD8-positive cells at days 91 and 154 p.i. (Fig. 4f).

All cats in group I and II sero-converted and developed neutralizing antibodies (Fig. 5a–d). Antibodies detectable by IFT appeared 14 days p.i. in group II and 25 days p.i. in group I. From day 25 and onwards, titers increased in both groups, reaching a maximum 119 days p.i. in group II and 154 days p.i. in group I (Fig. 5a, c). The CD4- and CD8-positive subpopulations increased synchronously with a slight predominance of CD4-positive T cells (Fig. 4f). The frequencies of T cells then declined, reaching a minimum 35 days p.i. At the same time, a significant increase in the frequency of B cells was observed. This switch in T and B cell frequencies was more pronounced in group II than in group I. During the remainder of the study, the frequencies of T cells increased and approached control values. However, a significant decrease in CD4-positive cells was observed at 63 and 154 days p.i., and a significant decrease in CD8-positive cells at days 91 and 154 p.i. (Fig. 4f).

Cyclophosphamide treatment

Treatment with cyclophosphamide at days 154 or 155 p.i. did not result in any apparent reactivation of the infection. The clinical condition of the animals remained unchanged. As shown by flow cytometry, the effect on frequencies of cells in lymphocyte subsets was not significant (Fig. 4a–f).

Discussion

The aim of the present study was to investigate the susceptibility of cats to BDV, using two different strains of virus: the rabbit-adapted BDV strain V of horse origin [19] and the newly isolated feline BDV [24]. Since the natural route of infection in cats is unknown, we chose to inoculate the animals intracebrally to ensure viral access to the CNS. Of a total of eight inoculated cats, three developed definite neurological signs and encephalitis. Two of these were infected with BDV strain V and one with feline BDV. The distribution pattern of inflammatory lesions in the present cats considerably differs from that in natural BD of horses, as well as that in feline SD, both of which show predilection for the limbic system and the brain stem [9, 20]. One possible explanation for this may be the route of inoculation. As indicated by the periventricular localization of lesions in one of the cats inoculated with BDV strain V, spread of virus following intracebral administration probably occurred via the CSF and the ventricles. Another explanation for the atypical distribution pattern in the present cats could be that histopathological examination was carried out only during the chronic stage (between 5 and 6 months p.i.), when the inflammatory reaction would be expected to be less prominent. In contrast, cats with natural SD usually are killed and examined post mortem at the peak of clinical disease and encephalitis.

The presence of plasma cells close to neurons in all infected cats, including those without encephalitis, has previously been observed in natural cases of SD [23]. It is possible that this phenomenon might be associated with antibody-mediated clearance of virus from infected cells, such as has been described in Sindbis virus encephalitis of mice [17] and experimental rabies virus infection [8]. In this context, it is of interest that inflammatory brain infiltrates in experimental rabies of cats typically involve plasma cells in close association with neurons [27, 31]. It should be noted that the genomes of BDV and rabies virus contain homologous sequences [6, 7] and that BD and rabies also share several pathogenetic features [11].

The clinical picture in one of the BDV strain V-infected cats, including an acute stage characterized by behavioral and motor disturbances, and a chronic stage predominated by mental dullness, is reminiscent of the biphasic neurological syndrome observed in rats and tree shrews experimentally infected with BDV [13, 18, 34]. Interestingly, recovery from the acute stage of disease in this cat coincided with a rise in neutralizing antibody titers, a phenomenon previously observed in experimentally infected rats [18]. In the other two cats with neurological signs, a correlation between clinical recovery and the appearance of neutralizing antibodies was less clear. Neutralizing antibodies in BD are thought to be directed against the 18-kDa viral glycoprotein [12, 35], and have been shown to prevent BDV spread in infected cell cultures in vitro [19]. However, the functional role of such antibodies in vivo awaits further elucidation.
A comparison between the serological data and the flow cytometric results shows that the rise of serum antibodies detectable by IFT coincided with an increase of peripheral B lymphocytes in both of the infected groups. The early peripheral T cell increase present in cats infected with feline BDV, but not with BDV strain V, and the fact that the former cats sero-converted somewhat earlier than the latter ones, suggest that the feline virus exerted a more vigorous effect on the immune system. This could be due either to the higher titer of the feline virus or to different antigenic properties of feline BDV and BDV strain V.

None of the present cats developed the slowly progressive, paralytic syndrome typical of feline SD. They were all able to recover from the acute disease stage, and the inflammatory lesions in their brains at the time of death were less severe than the lesions usually observed in natural SD. It is possible that some of the differences between natural and experimental disease in cats may be dose related, as indicated by a recent study in rats inoculated intracerebrally with high-dose or low-dose attenuated BDV [30]. The high-dose animals rapidly eliminated the virus by an early and efficient immune response, while in the low-dose rats the immune response was delayed and viral replication therefore proceeded, causing severe encephalitis.

Furthermore, it should be noted that no neutralizing activity has so far been found in sera from cats with SD ([22] and unpublished observations). In contrast, all of the experimentally infected cats developed a strong cellular as well as humoral immune response, including neutralizing antibodies.

Nevertheless, despite a seemingly adequate immune response, some of the present cats failed to clear virus completely from the CNS, as evidenced by the presence of BDV-specific antigen and nucleic acid in certain brain regions. A limited viral replication apparently continued, providing the necessary antigenic stimulus for the maintenance of inflammation. It is of interest to note that BDV mRNA was present in the spleen, but not in the brain, of one of the cats. This is in accordance with previous studies, showing that BDV during later stages of infection will spread centrifugally via peripheral nerve fibers to many different organs [10]. The importance of this in terms of viral persistency, chronic carriers and shedding of virus calls for further investigations.

In conclusion, we have shown that cats infected intracerebrally with either BDV strain V or feline BDV may develop neurological signs and encephalitis of a non-fatal character. An early cellular as well as humoral immune response seems to be instrumental for survival and recovery. Clearly, further studies are necessary to understand the pathogenesis of BDV infection in cats. Of special importance would be to investigate the clinical and immunological outcome of the infection when other, more natural, inoculation routes (for instance intranasally) are used. However, the successful induction of neurological signs and encephalitis in one cat intracerebrally inoculated with feline BDV, together with the detection of BDV antigen and nucleic acid in the brain of this cat, provides strong evidence for the notion that BDV is the etiological agent behind feline SD.

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