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Studies of In Vivo Distribution of Bovine Herpesvirus Type 4 in the Natural Host

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The in vivo distribution of bovine herpesvirus type 4 (BHV-4) was examined by testing nasal and conjunctival exudates, peripheral blood leukocytes, and various organs of experimentally infected calves. For virus detection, a nested PCR assay, virus isolation, and immunohistochemistry were applied. The nervous system and the muscles were free of viral DNA. Liver and intestinal lymph nodes contained low amounts of virus (less than two copies per 1 μg of cellular DNA). Intestinal, tonsil, thymus, and kidney tissues contained more viral DNA copies (5 to 50 copies per 1 μg of cellular DNA). The highest amounts of BHV-4 DNA (50 to 500 copies per 1 μg of cellular DNA) were found in the spleen, lungs, trachea, and nasal epithelium. Amplification of DNA from blood lymphocytes through postinoculation (p.i.) day 48 proved that the virus started to replicate in these cells immediately after inoculation of the calves and that intensive virus growth took place during the 7 to 8 weeks of the infection. The number of virus-infected lymphocytes reached the maximum on p.i. days 22 to 26 and slowly declined thereafter. Virus-infected cells were found only in the spleen on p.i. day 48 by immunohistochemistry. Western blotting (immunoblotting) detected signs of an immune response against 9 of the 29 BHV-4 proteins.

Bovine herpesvirus type 4 (BHV-4) represents a large number of antigenically related viruses. The first strain of BHV-4 was isolated in 1963 in Hungary from calves showing clinical signs of respiratory disease and keratoconjunctivitis (2). Isolation of different strains has been reported from animals showing a variety of clinical signs, such as conjunctivitis, pneumonia, inflammation of the upper respiratory tract (21), metritis (27), skin lesions (14), ulcerative mammillitis (28), enteritis (11), and tumors of the urinary bladder and rumen (15). Viruses specified as BHV-4 were also detected in apparently healthy animals (5).

On the basis of genome structure analysis, BHV-4 was recently classified as a gammaherpesvirus (29). The pathogenesis of this slowly growing herpes virus (20) is not yet fully understood. The main targets for viral replication are the lymphoid organs, the upper respiratory tract, and the urogenital and alimentary tracts. The lymphoid organs and mononuclear blood cells are also possible primary sites of viral latency (24), and the nervous system was also suspected of being involved in the persistence of the virus (18).

Despite the wide range of clinical symptoms, the etiological role of BHV-4 is not clear. Virus neutralization is not a suitable method for antibody detection (33). BHV-4-specific antibodies are demonstrated by complement fixation, dot immunobinding assay, agar gel immunodiffusion assay, immunofluorescence assay, or enzyme-linked immunosorbent assays (ELISAs) (10, 13, 32). The direct detection of the virus is based on virus isolation and characterization of specific letter-shaped inclusion bodies (1).

DNA hybridization has also been used as a diagnostic tool (12). Ten picograms of purified viral DNA was detected by using 11- and 5.7-kbp *Hin*dIII fragments of BHV-4 as probes.

To detect BHV-4 from samples of organs from experimentally infected rabbits, a single PCR assay was developed by using the sequence data of the *Eco*RI-L fragment of viral DNA. This assay was able to detect 100 fg of viral DNA (approximately 6,000 virus particles). These primers cross-reacted with bovine cells; thus, they were not suitable for the detection of BHV-4 in specimens of bovine origin (22).

In order to shed more light on the etiological role of BHV-4, novel approaches of virus detection are needed. The aim of the present study was to examine the in vivo spread of BHV-4 by applying a nested PCR as a highly sensitive method of viral DNA amplification, as well as immunohistochemistry, Western blotting (immunoblotting), and virus isolation. These experiments provide novel information on the distribution and possible pathogenic role of BHV-4 in its natural host.

MATERIALS AND METHODS

Experimental animals. Twelve calves (age, 7 days) of the Holstein-Friserian breed, all seronegative for BHV-4 as determined by ELISA, were used in the experiment. All calves were born to animals in the same BHV-4-seronegative herd and were placed in isolation units immediately after delivery. They were fed colostrum for the first 3 days; thereafter, they were fed Puri-lac milk powder (Inntaler Mischfutter GmbH & Co. KG, Wasserburg, Germany) and alfalfa hay.

Experimental inoculation. Nine calves (calves 1 to 9) were inoculated with cell-free BHV-4 supernatant (titer, 10^6 PFU/ml) by injecting 20 ml of virus intranasally (10 ml to each nostril) and 20 ml intratracheally via a 20-gauge needle at a level 10 cm below the larynx. Calves 10 to 12 were mock-infected negative controls. The control calves were inoculated as described above, but uninfected cell culture fluid containing 8% inactivated bovine serum was used. The animals were kept separated in isolation rooms.

Virus. The seventh passage of BHV-4 strain Movár 33/63 isolated by Bartha et al. (2) was used to inoculate the calves. The virus was propagated on bovine primary testicle cells and on a bovine embryonic lung cell line (EBL cells) (30). The cells were maintained in minimal essential medium (MEM; Serva, Heidelberg, Germany) containing $NaHCO_3$ supplemented with 8% newborn calf serum, 0.34 g of L-glutamine (Sigma, St. Louis, Mo.) per liter, 500,000 IU of

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TABLE 1. Isolation of BHV-4 from nasal, conjunctival, PBL, and various tissue specimens

p.i. day	Isolation of cell-free virus[a]			Isolation of cell-associated virus by cocultivation							
	Nasal specimens	Conjunctival specimens	PBL	Lungs	Spleen	Liver	Thymus	Muscle	T.g.[b]	M.o.[c]	Cortex
3	8/9	6/9	7/9	+	+	+	+	−	−	−	−
6	8/8	5/8	5/8	+	+	−	+	−	−	−	−
9	7/7	4/7	5/7	+	+	−	+	−	−	−	−
12	2/6	1/6	2/6	+	−	−	−	−	−	−	−
15	0/5	0/5	0/5	+	+	+	−	−	−	−	−
21	0/4	0/4	0/4	+	+	−	−	−	−	−	−
28	0/3	0/3	0/3	+	+	+	−	−	−	−	−
35	0/2	0/2	0/2	+	+	+	−	−	−	−	−
48	0/1	0/1	0/1	+	+	−	−	−	−	−	−

[a] Values indicate number of calves from which virus was isolated/total number of calves tested.
[b] T.g., trigeminal ganglia.
[c] M.o., medulla oblongata.

penicillin per liter, and 0.5 g of streptomycin-sulfate per liter. The virus was harvested at 80% cytopathic effect, and the cell culture fluid was centrifuged at 1,860 × *g* for 15 min.

Clinical observations. Clinical observations of inoculated and control calves were carried out daily by recording rectal temperatures and all signs and symptoms of the infection.

Experimental design and collection of specimens. The inoculated calves were killed during the first 2 weeks at 3-day intervals; thereafter, one calf was killed each week. The control animals (calves 10 to 12) were killed on postinoculation (p.i.) days 15, 28, and 48, respectively. Every third day nasal and conjunctival swabs and blood samples were taken from all of the animals for virus isolation. Eight organs of the animals that had been killed were used to prepare cocultures (Table 1).

Because of the large number of samples, the organs and lymphocytes of one control calf and one inoculated calf killed on p.i. day 48 (calves 9 and 12, respectively) were selected for the PCR and immunohistochemistry studies. The following organs were tested: spinal cord, brain, heart and skeletal muscle, liver, lymph nodes, thymus, kidney, small and large intestines, tonsils, lungs, spleen, trachea, and nasal epithelium (Tables 2 and 3).

The leukocytes collected from calves 9 and 12 every second day for PCR assay were purified by Ficoll-Paque separation (Pharmacia, Uppsala, Sweden) and counted, and suspensions containing 10^6 cells per 5 μl were used for PCR studies. Ficoll-Paque separates the granulocytes and lymphocytes; only the lymphocyte fraction was tested by PCR (6). Once a week serum samples were also collected from calves 9 and 12 for Western blot analysis. All specimens collected for virus isolation were used immediately, while tissue, lymphocyte, swab, and serum samples for other assays were stored at −70°C prior to testing.

Isolation of cell-free virus from nasal conjunctival swabs and PBL specimens. For cell-free virus isolation, the nasal and ocular swab samples were immersed in MEM containing antibiotics at concentrations three times higher then that described for cell cultures and were vigorously shaken and centrifuged at 10,500 × *g* for 20 min, before the inoculation of 0.1 ml into the cell cultures. Peripheral blood leukocytes (PBLs) were frozen and thawed three times and were centrifuged as described above; the supernatant was then inoculated onto confluent cultures of EBL cells.

Isolation of cell-associated virus from tissues by cocultivation. The organ pieces were washed in MEM, cut into 1- to 5-mm^3 pieces, and treated with 0.25% trypsin at 37°C until turbidity was observed. All of the visible particulate material was discarded, and the supernatants, which were rich in single cells, were cooled and centrifuged at 150 × *g* for 10 min. The sedimented cells were suspended in MEM with antibiotics and 8% fetal calf serum and were mixed with an equal proportion of the EBL cell suspension. The mixed cell suspensions were cultured on 24-well tissue culture plates (Greiner, Frickenhausen, Germany) in an incubator with a humidified 5% CO_2 atmosphere at 37°C. Cultures exhibiting any kind of cell degeneration were stained with hematoxylin-eosin. Preparations showing intracellular inclusion bodies characteristic of BHV-4 (1) were considered positive. Fluids of negative cultures were subpassaged five times.

Production of monoclonal antibodies. BALB/c mice were subcutaneously inoculated with the Movár 33/63 strain, which had been purified by ultracentrifugation and mixed with complete and incomplete Freund's adjuvant. The mice were immunized at 2-week intervals until the BHV-4-specific antibody titer reached 1/2,000 by ELISA. The fusion procedure was carried out as described by Köhler and Milstein (17) with SP2 myeloma cells and 50% polyethylene glycol 1500 (Carl Roth GmbH, Karlsruhe, Germany).

Immunohistochemistry. For immunohistochemistry, we followed the method described by Drén and Németh (9), with slight modifications. Instead of polyvalent serum, a pool of three monoclonal antibodies and rabbit anti-mouse immunoglobulin G (Dakopatts, Gostrup, Denmark) were used. The slides were counterstained with hematoxylin. The sensitivities of the monoclonal antibodies were checked by ELISA. The antibodies could detect 1.5 μg of purified BHV-4 proteins. All of the PCR-positive organs of calf 9 and, as negative controls, the same organs of mock-infected calf 12 were examined by immunohistochemistry.

PCR. A nested PCR assay has been developed by selecting primers from the thymidine kinase gene of BHV-4, considering the characterization of this genomic region by Lomonte et al. (19). The sequences were obtained from the GenBank EMBL data bank (accession number AC 49773). The following sequences were selected for use as primers: 5′-GTTGGGCGTCCTGTATGGT AGC-3′ (primer 1; positions 132 to 153), 5′-ATGTATGCCCAAAACTTATA ATATGACCAG-3′ (primer 2; positions 669 to 698), 5′-TTGATAGTGCGTT GTTGGGATGTGG-3′ (primer 3; positions 339 to 363), and 5′-CACTGCCCG GTGGGAAATAGCA-3′ (primer 4; positions 577 to 598). The outer primers (primers 1 and 2) flanked a 567-bp fragment; the inner primers (primers 3 and 4) amplified a 260-bp product. The oligonucleotides were synthesized in a PCR Mate DNA synthesizer (Applied Biosystems, Warrington, United Kingdom). The PCR amplifications were carried out in 50-μl reaction mixtures containing 5 μl of 10× PCR buffer (100 mM Tris [pH 9.0], 500 mM KCl, 1 mg of bovine serum albumin per ml), 100 μM (each) deoxynucleoside triphosphate (Pharmacia Biotech), 15 pmol of each primer, 1 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 5 μl of 25 mM $MgCl_2$, and 5 μl of sample (maximum of 0.9 μg of total DNA in the reaction mixture; see Table 2). The aqueous phase was overlaid with 2 to 3 drops of mineral oil (Sigma). The amplification was performed in a Biometra TRIO-thermoblock (Biometra, Göttingen, Germany). The first five amplification cycles included denaturation at 94°C for 45 s, annealing at 56°C for 1 min, and synthesis at 72°C for 1.5 min. Subsequently, an additional 25 cycles of 94°C for 45 s, 51°C for 1 min, and 72°C for 1.5 min were performed. After the last cycle the tubes were kept at 72°C for 7 min to complete the extension. Finally, the mixtures were cooled to room temperature. DNA extracted from purified BHV-4 and distilled water served as positive and negative controls, respectively, in each PCR assay.

Electrophoresis of the PCR products. The PCR products were analyzed by electrophoresis in 2% agarose gels by using 0.5× Tris-borate-EDTA as the running buffer (31). The ethidium bromide-stained bands were visualized with UV light, and the bands were recorded with a video camera (Cybertech, Berlin, Germany). The molecular sizes of the fragments were compared with those of a 100-bp ladder (Gibco Europe, Paisley, Scotland).

Specificity of the PCR. The specificity of the PCR was evaluated by testing the following herpesviruses: BHV-4 (23 strains, including Movár 33/63), BHV-1 (11 strains, representing subgroups 1.1 and 1.2); BHV-2, -3, and -5 (one strain each); suid herpesvirus type 1 (SHV-1, pseudorabies virus; three strains); and equine herpesvirus types 1 to 4, feline herpesvirus, and canine herpesvirus (one strain each). All animal herpesviruses originated from our own collection (A. Bartha). DNA preparations of six human herpesviruses (herpes simplex virus types 1 and 2, human cytomegalovirus, Epstein-Barr virus, varicella-zoster virus, and human herpesvirus 6) were kindly provided by T. Bergström, Gothenburg, Sweden. In order to check the specificity of the PCR, the bovine cell genome was also tested (EBL cells).

Sensitivity of the PCR. For sensitivity studies, BHV-4 DNA was used. Virus strain Movár 33/63 was propagated in EBL cells, the virions were purified with a metrizamide gradient and treated with proteinase K (Sigma), and then the DNA was extracted by the phenol-chloroform method as described previously (31). The concentrations of the extracted DNA were measured with a spectrophotometer (GeneQuant RNA/DNA Calculator; Pharmacia Biotech), and the number of BHV-4 DNA copies was calculated (6.366×10^9 pure virus particles contains 1 μg of BHV-4 DNA). The DNA was diluted to obtain 1,000, 100, 10, 1, and 0.1 genome copies in 5-μl volumes, and each dilution was tested by the PCR.

Detection of BHV-4 DNA in organs by the PCR. DNA was extracted from the different organs as described above, the concentrations were measured, and

TABLE 2. Detection of BHV-4 genome by PCR in the organs of the experimentally infected calf[a]

Organ	Assay result with the following amt (ng) of cellular DNA in reaction mixture[b]:					Estimated no. of virion copies/1 μg of cellular DNA
	900	500	200	20	2	
Spinal cord	−	−−				0
Brain	−	−−				0
Heart muscle	−	−−				0
Skeletal muscle	−	−−				0
Liver	+	−−−				< 2
Lymph node[c]	+	+	−−−			2–5
Thymus	+	+	+	−−−		5–50
Kidney	+	+	+	−−−		5–50
Small intestines	+	+	+	−−−		5–50
Large intestines	+	+	+	−−−		5–50
Tonsil	+	+	+	−−−		5–50
Lungs	+	+	+	+	−−−	50–500
Spleen	+	+	+	+	−−−	50–500
Trachea	+	+	+	+	−−+	500
Nasal epithelium	+	+	+	+	+	≥500

[a] Calf 9, which was killed on p.i. day 48, was used.
[b] −−+, three PCR assays, two negative and one positive; −−−, three PCR assays, all negative; −−, two PCR assays, both negative.
[c] Mesenteric lymph node.

dilutions were prepared to contain 900 ng of DNA in 5-μl volumes for each PCR assay. The samples were diluted (see Table 2), and the dilutions were tested until the first negative result was obtained. The dilutions yielding negative results were retested three times in order to confirm the absence of the virus (see Table 2).

Detection of BHV-4 in lymphocytes by the PCR. The number of lymphocytes was determined in a Bürker chamber with trypan blue staining. The DNA was extracted from 10^6 lymphocytes as described above and was serially diluted and tested by the nested PCR. From the highest concentration that yielded a negative result, two more reactions were carried out (see Table 3).

Western blotting. Serum samples from animals 9 and 12, which were obtained weekly, were tested by Western blotting. The proteins of metrizamide gradient-purified BHV-4 were electrophoresed, blotted onto nitrocellulose paper in a Protean II xi Cell device (Bio-Rad Laboratories, Richmond, Calif.), and stained with 3-3′-diaminobenzidine tetrahydrochloride (DAB) as described by Sambrook et al. (31).

RESULTS

Clinical observations. Five infected calves showed clinical signs of bronchitis and continuous coughing, and a slight nasal discharge appeared on p.i. days 3 to 4. In three infected animals, diarrhea was observed on p.i. days 5 to 8. From p.i. week 2, all of the clinical signs ceased, and only anorexia was observed. The body temperatures of all of the animals remained normal (below 39°C). The control animals were healthy and active throughout the experiment.

Virus isolation. Virus was isolated from the nasal and conjunctival swabs until the end of p.i. week 2. Thereafter, the conventional isolation method did not detect virus from the excretions. By cocultivation, cell-associated virus was detected throughout the experiment in samples from the lungs and the spleen but not in samples from the muscles and the nervous system tissues (Table 2). The isolation attempts required several passages in each case. No virus was isolated from samples from the control calves.

Immunohistochemistry. From the organ samples taken on p.i. day 48, the spleen was positive. Only a few spindle-shaped, elongated cells parallel to each other (two to three together) were stained by the monoclonal antibodies.

Specificity of the PCR. Both primer pairs reacted with all 23 BHV-4 strains tested (including Movár 33/63). The primers did not react with any of the other herpesviruses or with DNA from the bovine genome. The only exception was Epstein-Barr virus, which yielded a weak PCR product when more than 10^6 virus copies were used in the nested PCR (data not shown).

Sensitivity of the PCR. In a single PCR, both primer pairs amplified visible PCR products from approximately 100 copies of BHV-4 virions. In the nested PCR, by testing one viral DNA copy in each reaction tube, amplification was observed in three of four assays. In the three positive assays, the PCR products appeared as strong, clear, distinct bands, while the negative sample was completely blank. By testing 0.1 viral DNA copy per reaction tube, two tubes were positive of 10 assay (data not shown).

PCR of tissue samples. Viral DNA was not detected in the nervous system or in the muscles. The liver and intestinal lymph node presumably contained low amounts of viral DNA, because these tissues yielded a positive PCR signal only if at least 400 to 900 ng of total DNA was involved in the reaction (theoretically, two to five virus particles in 1 μg of total tissue DNA). Intestines, tonsils, thymus, and kidneys contained more viral DNA copies, providing positive results when 200 ng of DNA was tested (theoretically, 5 to 50 virus particles in 1 μg of total tissue DNA). The highest density of viral DNA was found in the spleen, lungs, trachea, and nasal epithelium, which were positive when 2 to 20 ng of DNA was tested (theoretically, 50 to 500 virus particles in 1 μg of total tissue DNA). Tissue samples from an uninfected calf were negative by PCR. For the results of BHV-4 DNA detection in organs, see Table 2. Viral DNA was continuously detected from 1,000 lymphocytes from p.i. days 16 to 38. The number of infected lymphocytes peaked on p.i. days 22 to 26, when viral DNA was detected in as few as 10 to 100 cells (theoretically, 10^4 to 10^5 virus copies in 10^6 lymphocytes). After p.i. day 38, the number of infected lymphocytes slowly declined (theoretically, 10^2 to 10^3 virus copies in 10^6 lymphocytes). The results of BHV-4 detection in blood lymphocytes are given in Table 3.

Western blotting. By Western blotting, nine viral proteins were detected in the serum of the tested infected calf. These proteins were classified into three groups according to their appearance and persistence after infection. A band of a 95-

TABLE 3. Detection of BHV-4 genome by the PCR in lymphocytes of the experimentally infected calf

p.i. day	Detection of BHV-4 among the following no. of lymphocytes[a]:						
	1×10^6	4×10^5	1×10^5	1×10^4	1×10^3	1×10^2	1×10^1
0	−						
2	+	−	−				
4	+	+	+	−−−			
6	+	+	−				
16	+	+	+	+	+−−		
22	+	+	+	+	+	+	−−−
24	+	+	+	+	++−		
26	+	+	+	+	+	+	++−
28	+	+	+	+	−−−		
30	+	+	+	+	+	−−−	
32	+	+	+	+	+	−−−	
34	+	+	+	+	+	−−−	
36	+	+	+	+	+	−−−	
38	+	+	+	+	+	−−−	
40	+	+	+	+	−−−		
42	+	+	+	+	+−−		
44	+	+	+	+	−−−		
46	+	+	+	+	+−−		
48	+	+	+	−−−			

[a] See note *b* of Table 2.

kDa protein was strong early during the infection; however, it disappeared after p.i. day 34. Six proteins (190, 68, 51, 47, 18, and 16 kDa) were continuously detected throughout the experiment. Bands of proteins of 68 and 47 kDa remained strong, while a protein of 51 kDa became weaker in the later phase of the infection, and the three other proteins were continuously weak, but detectable. Two proteins (71.5 and 44 kDa) appeared as strong bands only after p.i. day 21. In the serum sample taken before virus inoculation and samples taken between p.i. days 14 and 21, no virus-specific proteins were detected. Western blotting was also negative for the serum sample from the uninfected calf (calf 12).

DISCUSSION

The aim of the present study was to obtain more information on the distribution and outcome of BHV-4 infection in cattle, the natural host of the virus. Our principle was to shed light on the in vivo spread of this agent of questionable pathogenicity by applying a nested PCR as a highly sensitive method of viral DNA detection, in parallel with immunohistochemistry for demonstrating viral proteins.

In general, the sensitivity of single PCR assays is inferior to that of nested PCR. Thus, we developed a nested PCR assay which proved to be highly specific and sensitive for BHV-4 detection. In order to ensure a high degree of specificity, primers were selected from the thymidine kinase gene region of the viral genome. It is well documented that viral thymidine kinase differs from animal thymidine kinase in its electrophoretic mobility and in its ability to utilize both CTP and ATP as phosphate donors (16, 19).

This PCR technique, in comparison with virus isolation and DNA hybridization, allowed us to examine more deeply the in vivo distribution of BHV-4 in the organs and blood lymphocytes of infected animals. By testing various dilutions of organ samples, the nested PCR allowed a rough estimation of the accumulation of viral DNA in various organs, even though it is not a quantitative assay. The high degree of sensitivity of the BHV-4 nested PCR allowed the detection of approximately one viral DNA copy. Considering the extremely high degree of sensitivity, one should consider that by testing suspensions with very low target molecule concentrations, there is a certain probability that no viral DNA will be pipetted into each reaction tube, and this will yield a negative PCR result. Simultaneous tests of several samples, theoretically with one virion copy in each sample, may yield both positive and negative results. Regarding the high degree of sensitivity, our intention was to determine the smallest amounts of total host nucleic acid of organs and blood lymphocytes which yield at least some positive results among the simultaneous reactions; i.e., the reaction mixtures contain approximately one viral DNA copy per PCR test. This approach allowed us to make a rather reliable estimation of the in vivo distribution of BHV-4 in the natural host.

It must be emphasized that all PCR studies were carried out by applying the precautions reported previously from our laboratory. Accordingly, false-positive results caused by contamination or carryover can practically be excluded, and the positive PCR results can be regarded as safe indications of the spread of the virus in the organs of the inoculated calves (3, 4).

The clinical signs observed in the calves confirmed that the epithelial cells of the upper respiratory tract are among the main targets and replication sites of BHV-4. The most severe clinical signs, which were observed during the first p.i. week, could be explained by the 3- to 4-day-long replication process of the virus prior to the production of specific antiviral antibodies. Regarding the diarrhea at p.i. days 5 to 8, we suppose that BHV-4 replication occurred in the intestinal mucosal epithelium, initiating diarrhea either directly or indirectly by contributing to bacterial invasion of the intestines. Unfortunately, we did not attempt to isolate virus from the intestines in the present experiment. However, the PCR clearly demonstrated the presence of viral DNA in both the small and large intestines on p.i. day 48 (Table 2). It is likely that the route of BHV-4 infection is typical of a herpesvirus, which primarily replicates in the epithelial cells of the respiratory tract and presumably also in the intestinal mucosal membranes.

Shedding of the virus was observed by virus isolation between p.i. days 1 and 12 in the nasal and conjunctival specimens and PBLs. Isolation of BHV-4 from nasal swab samples indicates the presence of infective BHV-4 particles in nasal epithelial cells, nasal exudates, or leukocytes of the respiratory mucosa. The present findings indicate the probability that after replication in epithelial cells of the respiratory tract the virus spreads to the intramucosal lymph nodes and is transported to various organs of the body by infected leukocytes. It is likely that at about p.i. days 10 to 14 the extracellular virions are eliminated by the humoral immune response. This is in agreement with the results of previous studies stating that BHV-4 has never been isolated from nasal and lung epithelial cells of experimentally infected calves after p.i. day 14 (7). The virus was continuously detected by PCR in blood lymphocytes, lungs, and spleens of infected animals during the 48-day experimental period. For example, by testing organ specimens from calf 9, which was killed on p.i. day 48, one can conclude that virus isolation and the PCR similarly demonstrated the virus in the lungs and the spleen. The two assays were also in agreement in that they detected no virus in muscles or nervous system tissues (Tables 2 and 3).

PCR results for organ samples indicate that the nervous system, muscles, and liver play no role in BHV-4 replication. The low level of viral DNA in the liver and the mesenteric lymph node could be explained by the presence of BHV-4-infected blood leukocytes and not just BHV-4-infected tissue cells. High amounts of viral DNA (about 50 copies per 1 μg of tissue DNA) were detected in organs of the immune system (spleen, tonsil, and thymus) and organs which are densely infiltrated with immune cells (kidney and intestines). On p.i. day 48, when the organs were collected, BHV-4 was found in 10^5 blood lymphocytes. Considering the relatively low number of virus-infected blood lymphocytes, the blood cells cannot be the source of 50 viruses in 1 μg of tissue DNA. These results indicate that immune cells are probably the main sites of BHV-4 replication and latency. The positive PCR results for very diluted samples of the nasal epithelium and the lungs might be explained by the fact that infection was initiated by the intranasal and intratracheal routes.

It should be emphasized that neither virus isolation nor the PCR could detect BHV-4 in nervous tissues. These data support the findings of Osorio et al. (25), who could not detect virus in the nervous system of BHV-4-infected rabbits. It is also possible that different strains of BHV-4 vary in their capacity to invade the nervous system. In that regard, BHV-4 represents a heterologous group of viruses, and further studies are required to study this question.

PCR revealed that the virus appeared in blood lymphocytes of the inoculated animals within 2 days p.i. The PCR data indicate that virus replication occurs in blood lymphocytes during the first 7 weeks of infection and that during p.i. weeks 2 to 5 the number of infected cells is very high. Our PCR data support the findings of Osorio and Reed (24), who found by virus isolation that BHV-4 causes lymphoid-associated persis-

tent infection. Since the number of infected lymphocytes started to decrease after p.i. day 38, it is likely that at this stage most of the virus-invaded cells were destroyed by the developing cellular immune response. The results proved that, contrary to the results of previous investigators (26), BHV-4 infects and probably replicates in blood lymphocytes and not (or not only) in macrophages. Such a long period of persisting virus-infected lymphocytes was found only in Epstein-Barr virus infections, which is a further proof of the close relation of BHV-4 and Epstein-Barr virus (8).

It is interesting that viral DNA was found in the nasal and tracheal mucosae of calf 9. This indicates that BHV-4 is able to replicate or is also able to develop latency in the upper respiratory tract. Even epithelial cells of the respiratory tract could be regarded as sites for viral latency, despite the fast division rate of these cells from 4 to 7 days. Recent studies documented the fact that Epstein-Barr virus replicates in the basal layer of the epithelial cells of the mouth, and secretion of virus was also detected. There is evidence that epithelial Epstein-Barr virus infection is the main factor in the persistence and production of Epstein-Barr virus (23). One can speculate that microscopic lymph nodes under the epithelial cells in the connective tissue might harbor latent virus. The infected lymphoid cells might be responsible for the positive PCR results on p.i. day 48 when testing the nasal, tracheal, lung, and intestinal epithelium. The fact that by immunohistochemistry virus-specific proteins were not observed on the surface of the cells in most of the tested organs, while BHV-4 DNA was detected by the PCR, indicates that BHV-4 probably was in latency on p.i. day 48, when the organs of calf 9 were collected.

Western blotting detected on p.i. day 48 antibodies specific for nine viral proteins. Even if the temporal host immune response may vary for the different antigenic components, these nine proteins might be among the most immunogenic ones. Detection methods based on these viral proteins should be developed for further studies of the distribution of BHV-4 in cattle.

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