

The humoral and cellular immune response of sheep against Borna disease virus in endemic and non-endemic areas

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Summary

Borna Disease (BD) is a mostly fatal disease of horses and sheep endemic in central Europe. Antibodies to Borna disease virus (BDV) have been described in sheep and other species living in BD non-endemic areas. Meaningful clinical BDV serology is hampered by difficulties in defining serological cut-offs, which require the investigation of populations from endemic areas. Here we studied BD serology in sheep from endemic and non-endemic areas of similar geography in Switzerland. Antibodies to BDV antigens were detected by ELISA and indirect immunofluorescence analysis (IFA) only in sera from 3 of 6 sheep with autopsy confirmed BD. One serum was positive by IFA but not by ELISA, while 2 sera were negative in both assays, indicating that not all diseased animals develop BDV specific antibodies. Six % of clinically healthy animals (6/106) from an endemic area and 2% from a non-endemic area (4/192) had serum antibody to either BDV p40 or p24 as detected by ELISA. None of the animals showed a cellular immune response to BDV p40. In some healthy sheep from the endemic area, serum antibody titers to BDV p24 antigen remained elevated over several months without onset of disease symptoms. Infections with either BDV or related viruses may thus occur at low frequency in sheep from non-endemic areas leading to the production of antibodies to BDV antigens. We further propose viral strain differences or environmental factor(s) may determine the clinical outcome.

Key words: Borna disease virus, sheep, serology, ELISA, epidemiology

Humorale und zelluläre Immunantwort gegen das Virus der Bornaschen Krankheit beim Schaf in endemischen und nicht endemischen Gebieten

Die Bornasche Krankheit (BD, Borna Disease) ist eine meist tödlich verlaufende Krankheit der Schafe und Pferde, die in eng umschriebenen Gebieten in Mitteleuropa endemisch ist. Interessanterweise wurden Antikörper gegen das Virus der Bornaschen Krankheit (BDV, Borna disease virus,) bei Schafen gefunden, die sowohl aus Endemie-, wie auch aus Nichtendemiegebieten stammten.

In der vorliegenden Studie wurde die serologische Reaktion von Schafen gegen BD in geografisch vergleichbaren endemischen sowie nichtendemischen Gebieten in der Schweiz untersucht. Antikörper gegen BDV Antigene wurden mittels ELISA und Indirekter Immunfluoreszenz (IFA) nur in drei von sechs Schafen mit pathologisch abgesicherter BD gefunden. Ein Serum reagierte positiv im IFA aber nicht im ELISA, während zwei Seren in beiden Tests negativ waren, was darauf hindeutet, dass nicht alle erkrankten Tiere BDV-spezifische Antikörper entwickeln. Im ELISA zeigten 6% der untersuchten klinisch gesunden Schafe (6/106) aus dem Endemiegebiet und 2% aus dem Nichtendemiegebiet (4/192) Serumantikörper, die entweder gegen BDV p40 oder p24 gerichtet waren. Keines der untersuchten Tiere zeigte eine zelluläre Immunantwort gegen BDV p40. Bei einigen gesunden Tieren aus dem Endemiegebiet blieben die Serumantikörperspiegel gegen BDV p24 über mehrere Monate erhöht, ohne dass diese Tiere klinische Symptomatik gezeigt hätten. Möglicherweise kommen Infektionen, entweder mit BDV oder mit verwandten Viren, in einer niedrigen Frequenz bei Schafen in Nichtendemiegebieten vor, was zu einer Produktion von Antikörpern gegen BDV spezifische Antigene führt. Im weiteren könnten Unterschiede zwischen Virusstämmen oder Umweltfaktoren das klinische Bild bestimmen.

Schlüsselwörter: Bornasche Krankheit, Schaf, Serologie, ELISA, Epidemiologie

Introduction

Borna disease (BD) is a fatal neurological disease characterized by a progressive nonpurulent meningoencephalitis that is predominantly observed in horses and sheep (Dürwald and Ludwig, 1997). The causative agent of BD is an enveloped virus with a single-stranded RNA genome of negative polarity, the Borna disease virus (BDV). The viral genome contains information for six open reading frames (ORFs) (Cubitt et al., 1994; Briese et al., 1994; Pringle, 1997; Kraus et al., 2001).

After BDV infection, meningitis and encephalitis may develop paralleled by severe depression, ataxia, circular movements, dysphagia and other neurological malfunctions (Rott and Becht, 1995). In the absence of severe neurological malfunctions, infections with BDV are difficult to detect because reliable *in vivo* diagnostic tools are not available (Caplazi et al., 1999). Therefore, histopathology and immunohistology are still the most reliable methods to verify a clinical suspect. It is widely accepted that tissue damage in the brain is not caused by the virus itself but by an immunopathological reaction (Narayan et al., 1983; Bilzer et al., 1995; Stitz et al., 2002). In the rat model it has been shown that CD8+ T cells mediate the disease (Bilzer and Stitz, 1994; Noske et al., 1998; Planz et al., 1995; Stitz et al., 1992).

Historically, BD was reported to be restricted to horses and sheep living in certain areas of Germany, where the disease occurred frequently, causing hundreds of deaths per year (Dürwald, 1993). Presently, only isolated cases of BD are observed in these species in the endemic areas of Germany, Liechtenstein, Austria and Switzerland (Caplazi et al., 1999; Dürwald, 1993; Goetzmann, 2000; Rohner-Cotti, 1992; Suchy et al., 2000). However, natural infections of rabbits (Metzler et al., 1978; Otta and Jentzsch, 1960), cattle (Caplazi et al., 1994; Okamoto et al., 2002a), goats (Caplazi et al., 1999), felids (Degiorgis et al., 2000; Johansson et al., 2002; Lundgren et al., 1993), dogs (Weissenbock et al., 1998; Okamoto et al., 2002b), ostriches (Malkinson et al., 1993), donkeys and mules (Altmann et al., 1976; Caplazi et al., 1999) have been reported recently, even outside the classical endemic areas. In addition, Borna specific antibody and BDV-RNA have been found in humans and other species worldwide (Bechter, 1999; Bode et al., 1988; Bode et al., 1995; Chen et al., 1999; Nakamura et al., 2000; Rott et al., 1985; Schwemmle, 1999), and the discussion whether BDV infection is related to psychiatric disorders is still ongoing (Carbone, 2001; Staeheli and Lieb, 2001; Richt and Rott, 2001). Some data suggested that BDV is geographically more widely distributed than originally thought and that inapparent infections in species other than horses and sheep seem to be a common event. However, determination of BDV-specific antibodies and

detection of BDV-RNA was difficult to reproduce in different laboratories (Schwemmle, 1999).

The conditions of natural exposure of sheep to BDV that leads to infection of the CNS and to disease are not understood and the epidemiology of BD in endemic areas including the occurrence of single cases with a seasonal peak in spring and early summer remains to be elucidated (Dürwald, 1993; Carbone et al., 1987; Morales et al., 1988; Rott and Becht, 1995). In order to better understand the role of sheep in the epidemiology of BD, we performed a seroepidemiological study in sheep living in endemic and in non-endemic areas. Sheep from the endemic area were also tested for cellular immunity against BDV by delayed type hypersensitivity reaction (DTH) using recombinant p40 as antigen and by an interferon gamma assay (Roethel et al., 1990).

For the serological study we adapted our recently developed ELISA technology (Baumann et al., 1998) that exploits the high affinity interaction between the albumin-binding domain of streptococcal protein G (ABP) and rat serum albumin (RSA) to indirectly immobilize protein to solid phase (Akerstrom et al., 1987; Baumann et al., 1998; Nygren et al., 1990). For our application, each of the two viral proteins p40 and p24 were fused to ABP and expressed in the periplasmic space of *E. coli*, where the protein is folded and disulfide bond formation occurs (Power et al., 1992; Skerra and Pluckthun, 1988). The purified recombinant p40- or p24-ABP fusion protein was each indirectly immobilized on RSA precoated 96 well plates that allows binding of defined amounts of ABP fusion proteins under conditions that preserve the native structure of the immobilized ligand and thus maintain antigenicity (Baumann et al., 1998; Suter and Butler, 1986).

The novel ELISA approach offers the possibility to detect serum antibody, which would possibly escape other serological assays based on denatured antigen (Suter and Butler, 1986). We first analyzed mouse sera from animals experimentally infected with BDV. In the next step, we made use of the favorable situation in Switzerland that has endemic and non-endemic areas to (i) perform a comparative serological study on BDV, (ii) analyze the cellular immune response by DTH and (iii) observe sheep with antibody to BDV antigen over several months.

Animals, Materials and Methods

Construction of plasmids and expression of recombinant proteins

ORF I of BDV encoding p40 and ORF II encoding p24 as well as the gene encoding the 10 amino acid c-myc tag peptide (amino acid sequence: EQK-LISEEDL) used as control peptide in ELISA were each

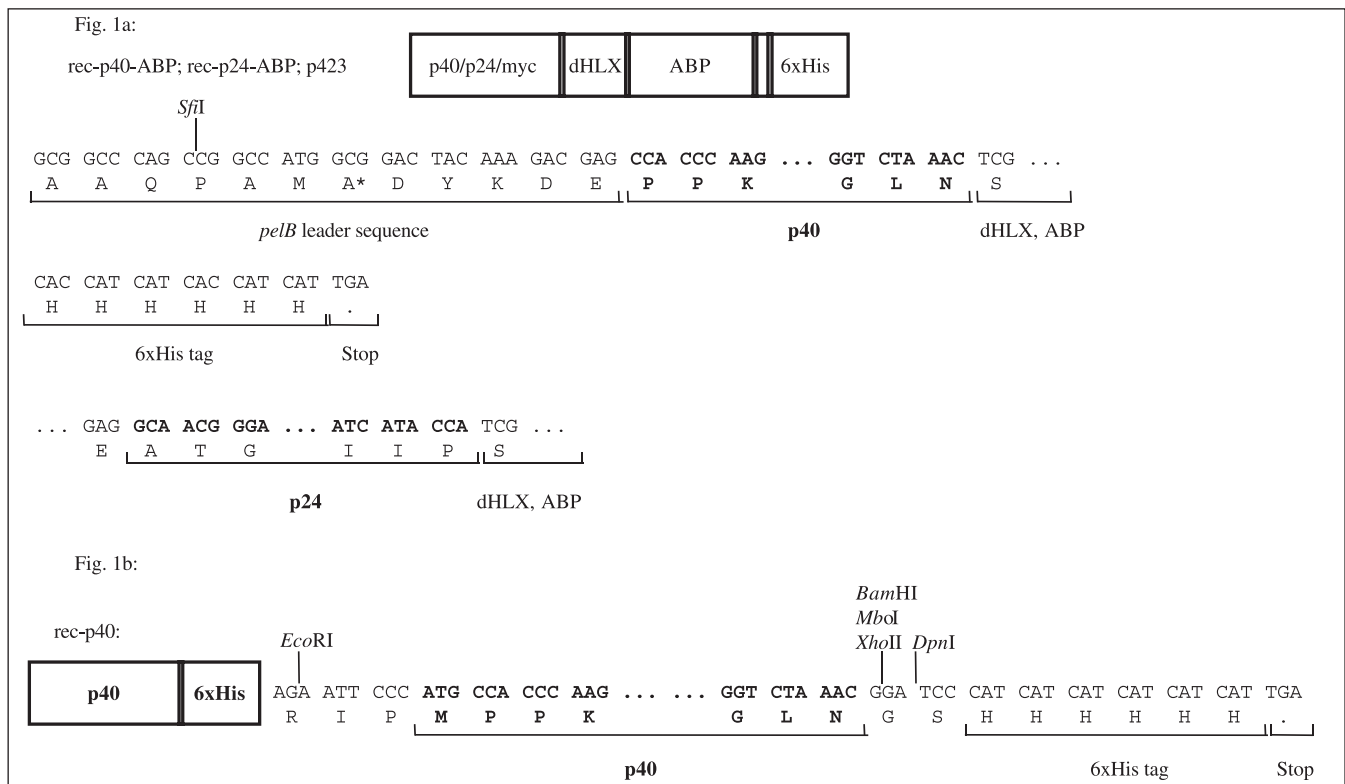


Figure 1a: Recombinant proteins used as antigen in the ELISA. BDV p40 and p24 each fused to the double helix module (dHLX), ABP and the 6xHis tag were expressed in *E. coli* and used for indirect immobilization on RSA precoated ELISA wells. The control protein p423 contains the c-myc tag peptide (EQKLISEEDL) coexpressed with the dHLX-ABP-6xHis-tag-cassette. Cleavage of the leader peptide takes place before or after the alanin (A*) residue.

Figure 1b: Recombinant p40 used in DTH experiments. BDV p40 was fused to the 6xHis tag and expressed in *E. coli*.

cloned into the expression vector pSB411 that allows production of fusion proteins containing the dHLX-ABP-6xHis-tag cassette (Baumann et al., 1998) (Fig. 1a). For DTH experiments (see below) the gene encoding viral protein p40 fused to a 6xHis-tag was cloned into the vector pETR247 (Fig. 1b). Expression of recombinant proteins rec-p40-ABP, rec-p24-ABP and p423 and purification by Nickel affinity chromatography used for ELISA was essentially as described (Baumann et al., 1998).

Expression of the recombinant protein rec-p40 used for DTH reaction (see below) was done in *E. coli* strain XL1blue (Bullock et al., 1987). Centrifugation sedimented bacteria from a 12 l culture were disintegrated in a french press (SLM Aminco French pressure cell press) at a pressure of approximately 1000 psi. Recombinant protein present in the supernatant was enriched by Nickel-NTA chromatography and further purified by anion exchange chromatography. For this, the Nickel-NTA-isolated protein was dialyzed against phosphate buffer (0.013 M NaH₂PO₄, 0.087 M Na₂HPO₄, pH 7.6) twice during 4 hrs at 4°C. Anion exchange chromatography was done with the Resource Q column (column volume 1 ml) (Pharmacia). First the column was precycled with both phosphate buffer, pH 7.6 (buffer A) and phosphate buffer containing 1M NaCl (buffer B) and then equilibrated with buffer A at 0.5 ml/min. The protein sample was applied

to the column and the column was washed with 5 ml of buffer A at 0.5 ml/min before elution started. Elution was done with a linear gradient up to 400 mM NaCl (=40% of buffer B). 50 fractions of 500 µl each were collected and analyzed by dot blot for the presence of the purified protein using a monoclonal antibody against BDV p40 (clone 5A10) (F.A. Grässer, unpublished data). Rec-p40 was eluted at a concentration of about 150 mM NaCl. Fractions that were positive in the dot blot analysis were separated on 10% SDS-PAGE and their purity was judged after Coomassie blue staining and western blot analysis. Fractions with only one band of about 40 kDa on Coomassie blue stained gels were pooled, the pool was dialyzed against PBS and after addition of 10% glycerol stored at -80°C.

ELISA

RSA precoated 96 well plates (Anawa Laboratories, Wangen, Switzerland) were coated overnight with 300 ng of one of the purified recombinant proteins (rec-p40-ABP, rec-p24-ABP or p423) in PBS (phosphate buffered saline; 136.89 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, 8.10 mM Na₂HPO₄, pH 7.2) at 4 °C. The optimal amount of protein had been determined in previous experiments (Baumann et al., 1998). For this and all subsequent steps 200 µl per well were used.

On the next day the plates were washed 3 times with PBS, 0.05% Tween 20 and once with PBS. Then the plates were blocked for 1 hr at 37 °C with 2 % BSA in PBS 0.05% Tween 20. In the meantime the sheep sera were diluted 1:100 in PBS and preincubated on another RSA plate to reduce background signals. After washing the blocked plates the sera were filled into the wells and the plate was incubated for another 2 hrs at 37 °C. After another washing step the secondary antibody conjugate was added at a dilution of 1:20000 (Alkaline-Phosphatase-conjugated-donkey-anti-sheep-IgG, Sigma, product no. A-5187). After incubation at 37 °C for 1 hr and further washing the substrate was added. For this 5 mg pNPP (p-Nitrophenylphosphate, phosphatase substrate 5 mg tablets, Sigma) were dissolved in 3 ml phosphatase developing buffer (1 ml diethanolamine, 25 µl 0.2 M MgCl₂, aqua bidest. ad 10 ml, pH 9.8). The substrate was filled into the wells and the plates stored in the dark. The OD was measured at 405 nm using a spectrophotometer (Anthos reader 2001, Anthos Labtec Instruments). Measurement was performed when the positive control serum had reached an OD₄₀₅ of 1.5 against rec-p40-ABP.

All sera were tested against each of the 3 recombinant proteins rec-p40-ABP, rec-p24-ABP, or p423 in duplicate and the mean OD was used to calculate the Signal-Background-ratio (SBR) for each antigen and each serum. For this the mean OD obtained by ELISA for p40 or p24 was divided by the OD obtained for the control protein p423.

Delayed type hypersensitivity reaction (DTH)

10 µg of rec-p40 in 100 µl PBS were ultrafiltered (pore size 0.45 µm) and injected intradermally into the skin on the medial side of the left thigh with a 25G needle. On the right thigh 100 µl of PBS was injected as control. After 4 to 6 and 48 to 72 hrs the application site was controlled for signs of inflammation, like swelling and erythema. The thickness of the skin at the application site was measured with a standard caliper.

Interferon gamma assay

Lithium heparin blood from sheep was collected in 7 ml vacutainer tubes and processed within 12 hrs. 1.5 ml of blood were incubated in 24 well cell culture plates with 30 µg of rec-p40 or p423 or 100 µl PBS for 20 hrs at 37 °C. The supernatant was harvested and analyzed for the presence of IFN gamma using a commercial test kit (Bovigam Bovine Gamma Interferon Test, CSL Limited, Parkville, Australia) (Rothel et al., 1990) following the manufacturer's instructions.

Sheep Sera

Serum samples of Borna diseased sheep

Serum samples of six Borna diseased sheep were examined for BDV-specific antibodies using our ELISA system. The clinical diagnosis of BD had been confirmed by histopathology in all cases and in five cases also by immunohistochemistry. One serum was obtained from Prof. L. Stitz (Bundesforschungsanstalt für Viruskrankheiten, Tübingen, Germany), the remaining five were obtained from Prof. F. Ehrensperger (Institute of Veterinary Pathology, University of Zurich, Switzerland).

Serum samples of sheep from the endemic region (principality of Liechtenstein)

106 serum samples from healthy sheep were collected at the abattoir. Tissues from these sheep had been analyzed by indirect immunofluorescence assay to detect antibodies against BDV and by histopathology, immunohistology and RT-PCR of brain sections to analyze the presence of BDV antigen or BDV-specific RNA, respectively (Goetzmann, 2000). In none of these animals Borna disease had been suspected clinically and none was positive by any other test (Goetzmann, 2000).

Serum samples of sheep from a non-endemic region

192 sera of sheep more than six months of age were collected in the cantons of Vaud and Valais during 1998. Serum from a twelve month old sheep was used as negative control for the ELISA. The sheep had been separated from his mother immediately after birth and was bottle raised without receiving colostrum. The sheep was born and housed in Zurich where BD is not endemic.

Serum samples from "borna- flocks" in Graubünden and Liechtenstein

Sera from 167 adult sheep originating from 6 flocks in Graubünden and Liechtenstein where BD had occurred ("borna-flocks") during the years 1999 and 2000 were analyzed for humoral immune response against BDV by ELISA and for cellular immune response by DTH and interferon gamma assay.

Negative control group for the DTH experiment

18 sheep from two herds kept at the University of Zurich were used as negative controls for the DTH experiment.

Statistical analysis of the ELISA results

Normal distribution

The different groups of samples were examined for normal distribution using the Lilliefors diagram (Iman, 1982; Lilliefors, 1967).

Test for statistically significant differences between two groups

The ELISA data from the different groups (non-endemic and endemic) were tested for a statistically significant difference using the test of Kolmogoroff and Smirnof (Kolmogoroff, 1933; Smirnof, 1939). P was chosen 0.05.

Analysis of correlation between data sets

A linear relationship between ELISA data and the reported age of the sheep and between p40 ELISA data and p24 ELISA data was analyzed by calculating the correlation coefficient and the Spearman rank correlation coefficient.

Results

Serological investigations

Analysis of virus-specific antibody in sera of Borna infected mice

There are contradictory data on BDV-specific serum antibody of sheep indicative for an ongoing or past infection. The consensus is that in general, BDV induces low amounts of antibodies and of low affinity. To avoid possible artifacts and interference with natural antibodies (Ochsenbein and Zinkernagel, 2000) three precautions were taken for ELISA (i): only purified, folded and graded amounts of recombinant p40 and p24 protein antigens were used, (ii): an ELISA system was chosen that minimizes denaturation of antigen (Suter and Butler, 1986; Baumann et al., 1998) and (iii): the ELISA was thoroughly tested with sera of mice (strain 129Sv/Ev) experimentally infected with BDV (strain He/80).

The purity of rec-p40-ABP and rec-p24-ABP antigens that were used in the ELISA system was verified by SDS PAGE and immunoblot. Only sera from the 35 BDV-infected mice reacted positively, whereas all sera from control mice scored negative (Fig. 2 and data not shown). Interestingly, all infected mice developed antibody to BDV p40 whereas only few developed antibody to BDV p24 although mice of a well defined genetic background were used. Therefore, for the analysis of sera from experimentally infected mice the sensitivity of the ELISA was high enough to achieve a diagnostic accuracy of 100 % for both negative and positive sera.

Analysis of virus-specific antibody in sera of Borna diseased sheep

Next, sera from sheep were analyzed. In a first step, sera of six sheep with natural BDV infection confirmed by post mortem analysis as well as one negative control serum were tested by ELISA. The signal-background-ratios (SBR) were calculated for each antigen using the

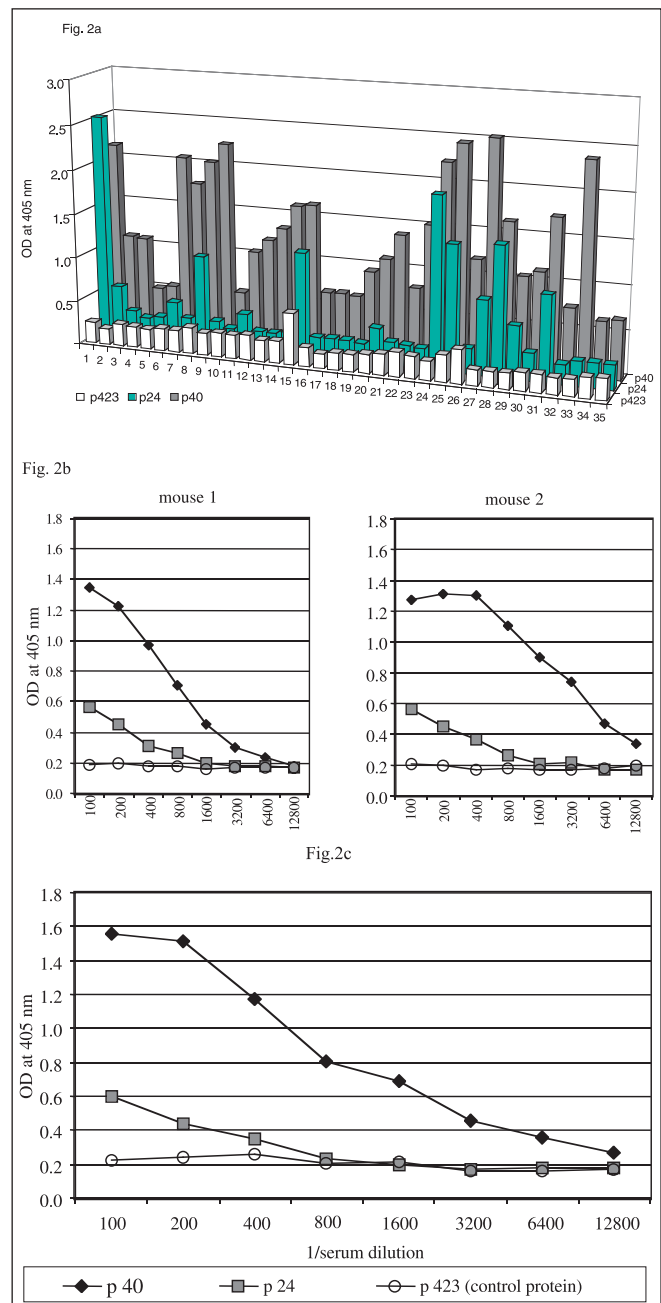


Figure 2: Serum antibody response to BDV p40, p24 or the control protein of experimentally infected mice (2a and b) or a naturally infected sheep (2c) determined by ELISA. (2a) ELISA results of 35 individual mice, all infected with BDV. Serum dilution 1:100. (2b) Curves of two randomly selected mice. (2c) Sheep no. 42.

OD₄₀₅ values obtained by ELISA analysis as described in materials and methods. SBRs determined for sera from known BDV-infected sheep varied between the individual sera (Tab. 1). The negative control serum (no.63) averaged at 1.06 ± 0.15 for p40 and 1.09 ± 0.15 for p24, indicating that serum from a healthy sheep did not react positively in the ELISA. Analysis of three sera from BDV-infected animals resulted in SBR values that were only marginally higher than those of the negative controls. For the remaining three sera the SBRs ranged between 2.34 and 7.47 indicating that some but not all BDV-infected sheep may have anti-

Serum no.	Diagnosis	SBR (p40)		SBR (p24)	
		average	standard deviation	average	standard deviation
3	BD	1.23		1.69	
7	BD	1.71		1.65	
14	BD	2.34		3.14	
153	BD	2.76		7.11	
268	BD	1.25		1.29	
42	BD	7.47	1.09 (=14.6%)	2.3	0.45 (=19.57%)
63	Healthy (negative control)	1.06	0.15 (=14.15%)	1.09	0.15 (=13.76%)

Table 1: Signal-background-ratios (SBRs) of 6 Borna diseased sheep and one negative control sheep. Sera were tested at a dilution of 1:100. Values of sera no. 42 and 63 represent the average calculated from 30 independent ELISA runs.

bodies specific for the recombinant viral proteins used in this ELISA.

Interestingly, the SBR determined for p40 or p24 for each individual serum varied markedly. For serum no. 42, a SBR of 7.47 was calculated for p40 whereas the one for p24 was only 2.3. By contrast, for serum no. 153 the SBR for p40 was 2.76 whereas the one for p24 was 7.11 (Tab. 1). We conclude that antibody of sheep infected with BDV may preferentially react against either p40 or p24.

Sera no. 42 and 63 were chosen as controls for all subsequent ELISA runs (see below). The interassay variation of the ELISA was between 13.8 and 19.6 % based on the determination of the SBR from 30 independent runs (Tab. 1). Thus, the ELISA was highly reproducible as shown for serum no. 42 and 63 (Tab. 1) or other sera analyzed at different occasions (data not shown).

The sera were also tested by indirect immunofluorescence assay (IFA) as described (Hallensleben et al., 1996). The results are shown in Table 2. Sera no. 42 and 153, which reacted positively in the ELISA, were pos-

Serum no.	Diagnosis	ELISA		IFA
		P40	P24	
3	BD	-	-	+
7	BD	-	-	-
14	BD	+	+/-	n.d
42	BD	+	+/-	+
153	BD	+	+	+
268	BD	-	-	-
63	healthy	-	-	-

Table 2: Comparison of the ELISA- and IFA- results of sera from Borna diseased sheep (sera no. 3, 7, 14, 42, 153, 268) and one negative control sheep (no. 63).

itive by IFA. Sera no. 7 and 268 as well as the negative control serum no. 63, which were negative by ELISA, were also negative by IFA. Serum no. 3 was positive by IFA, but negative by ELISA. Serum no. 14 was no longer available for further testing. Therefore, ELISA data and IFA data corresponded in 86% of the tested sera. Even though both test agreed favorably, the diagnostic value of these serological assays was only 50 %, because only three out of the six sera of diseased animals had antibodies against BDV antigens.

To further verify the specificity of the ELISA, we additionally tested sera from 106 sheep that had been shown to be negative for BDV antigen by immunohistology and RT-PCR (Goetzmann, 2000). In none of these sera antibodies to p24 or p40 could be detected by ELISA (data not shown). These data indicated that the ELISA is specific.

ELISA of sera collected from healthy sheep from endemic and non-endemic area:

In a next step, sheep sera from endemic and non-endemic areas of Switzerland and Liechtenstein were tested by ELISA. For this 167 serum samples from adult healthy sheep originating from 6 "borna-flocks" of the endemic areas were analyzed. A total of 192 sera of age-matched sheep from the non-endemic western part of Switzerland were used as reference.

The results are shown in Figure 3. The majority of SBRs calculated either for p24 or p40 remained below the level of 2 with the exception of a few sera for which SBRs of up to 3 were found for p40. For p24, SBR values higher than 3 were calculated for a few sera, the highest reaching the value of 6.2. For each of these sera, SBR was elevated only for p24, but not for both antigens. Repeated analysis of selected sera produced essentially the same results (data not shown).

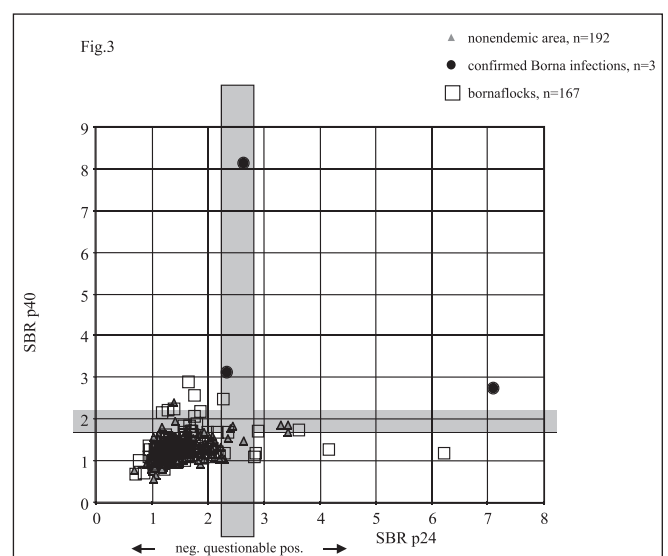


Figure 3: Correlation between ELISA results and age of sheep originating from bornaflocks. Sheep sera were tested for antibody to p40 and p24 at a serum dilution of 1:100. SBRs of p40 (crosses) and p24 (squares) were plotted against the age of sheep.

Among the 192 sera collected from sheep of the non-endemic area, the pattern of SBR was similar for p40 and p24 as calculated for the 167 sera from sheep living in endemic areas except that maximal SBR did not exceed 2.4 for p40 and 3.5 for p24, respectively (Fig. 3). Half of the 192 sera were randomly selected and retested with the same results (data not shown).

Definition of a cut-off point for positive/negative discrimination of BDV infection

The "classical" method to define a cut-off point is to test a negative reference population, to calculate the average and standard deviation from this group and to define sera with values higher than average plus twice the standard deviation as "questionable" and sera with values higher than average plus three times the standard deviation as "positive" (Greiner et al., 1994).

SBR values were calculated from ELISA data obtained from the 192 non-endemic sera and transformed into log values. Using the Lilliefors diagram we found that the transformed data set was normally distributed (data not shown). Using the transformed data from this negative reference population the following cut-offs were calculated:

p40-ELISA: sera with a SBR higher than 1.77 (log-value = 0.249) were defined "questionable"; sera with a SBR higher than 2.20 (log-value = 0.342) were defined "positive".

p24-ELISA: sera with a SBR higher than 2.22 (log-value = 0.346) were defined "questionable"; sera with a SBR higher than 2.83 (log-value = 0.452) were defined "positive".

Using the calculated threshold, ten sera (6%) of sheep from the endemic area, four sera (2%) from the control group of sheep living in non-endemic areas and three sera (50%) of sheep with confirmed BDV were "positive". However, there is no statistically significant difference ($p=0.05$) between the SBR data set of the endemic and the non-endemic group as analyzed by the test of Kolmogoroff and Smirnof (Kolmogoroff, 1933; Smirnof, 1939). Therefore, the statistical definition of a cut-off point as calculated here provides only a tendency of elevated frequency and possible amount of antibody with reactivity to rec-p40 or rec-p24 of sheep living in the endemic area when compared to those living in non-endemic areas. This tendency is further illustrated by frequency distribution graphs of the ELISA data (Fig. 4). In both groups the distribution of the data sets is positively skewed. The medians of the borna-flock group are 1.41 for p24 and 1.23 for p40, whereas that of the non-endemic group are 1.28 for p24 and 1.13 for p40, indicating that the data set of the former group is shifted to the right compared to the latter.

SBR for p40 and p24 are not correlated

The degree of linear relationship between p40 and p24 ELISA data from sera defined as „questionable“ or „positive“ was determined independently for sera of the borna-flock group and that of the non-endemic population. For this both the correlation coefficient and the Spearman rank correlation coefficient were calculated. The correlation coefficient was $r = -0.58$ (critical value = 0.44) for the borna-flock group and $r = -0.18$ (critical value = 0.55) for the non-endemic group. For the former group a negative correlation was calculated (absolute value of r is higher than the critical value), whereas for the latter ELISA values were not correlated (absolute value of r is less than the critical value). This further supports the finding, that p24 and p40 ELISA data are not positively correlated in individual sheep. Calculation of the Spearman rank correlation coefficient, which is based on the ranks of the data and not the data itself and is thus resistant to outliers, gave the same result (data not shown).

No correlation between SBR and age

To rule out the possibility that immunologically more experienced, older sheep preferably react positively in our assay, we analyzed our data for a possible correlation between seropositivity and age (data not shown). No correlation was found between the SBR for one of the viral antigens and the age of the sheep indicating that our assay is specific.

DTH

It was possible that a high SBR to p40 or p24 was correlated with specific cellular immune responses. To address this, we performed DTH experiments using highly purified rec-p40 and an ex vivo interferon gamma assay. DTH reactions as well as IFN gamma assays were negative in all 129 tested sheep from borna-flocks (data not shown).

Follow up study of seropositive sheep over several months

The three sera with the highest SBR value for p24 were all from the endemic area and they originated from the same borna-flock. These sheep were retested periodically for BDV-specific antibodies during a time period of four months. The individual data points are shown in Figure 5. Antibody titers remained at the same level in all three cases during the entire observation period. During the respective time frame no Borna cases were reported in this particular flock and all three sheep stayed healthy during the observation period. These data indicate that repeated serological testing of sheep from the endemic area is the only possibility to identify infected sheep, either during the incubation period or survivors without BD specific symptoms.

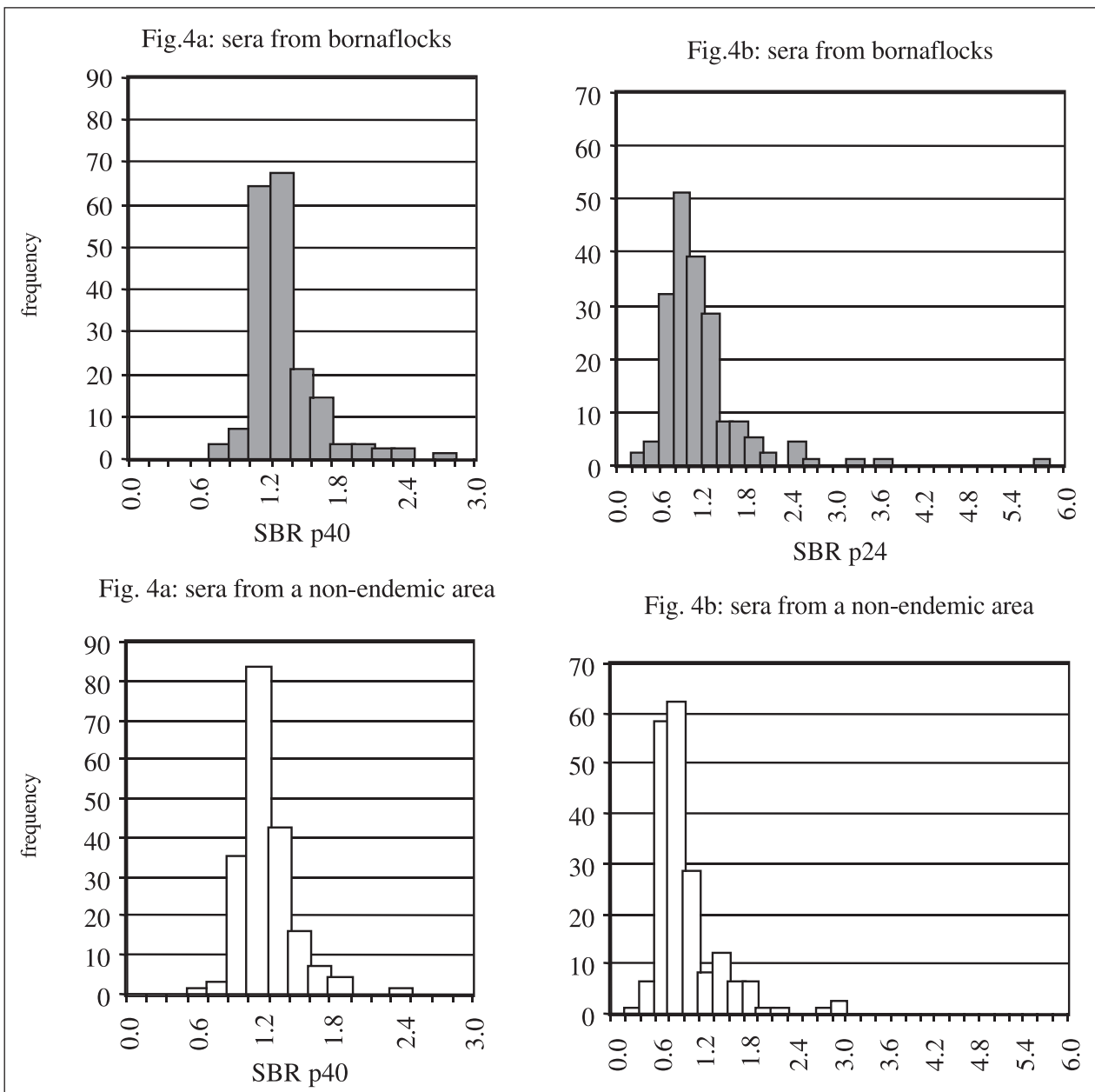


Figure 4: Frequency distribution of BDV ELISA results. Sheep sera originating from bornaflocks (gray bars, $n=187$) and from a non-endemic area (white bars, $n=192$) were tested for antibody to BDV p40 (a) and BDV p24 (b). SBR is plotted against frequency of occurrence.

Discussion

The aim of this study was to compare the presence of BDV-specific antibody and cellular immune response in sheep from endemic and non-endemic areas and to get information about possible subclinical BDV infections in sheep. For the serological study we made use of a newly developed ELISA technology where natively folded protein is used as antigen (Baumann et al., 1998). All mice experimentally infected with BDV produced antibodies to BDV antigen, whereas all control sera were negative. We thus concluded that the ELISA was sensitive, reproducible and highly specific. Next, we analyzed the sera of six histologically confirmed Borna diseased sheep of which only three

scored positive in our ELISA. In contrast, sera from more than 100 sheep histologically confirmed to be negative for BDV were also negative in ELISA further indicating the specificity of the assay. The fact, that some but not all Borna diseased sheep may have detectable virus-specific antibody (Tab. 1) was noted previously (Caplazi and Ehrensperger, 1998; Dürrwald, 1993; Grabner and Fischer, 1991; Lee, 1989; Ludwig and Thein, 1977; Metzler et al., 1976; Rohner-Cotti, 1992). The ELISA data and the IFA data of Borna diseased sheep correlated to 86%. Therefore, the low prevalence or absence of detectable serum antibody in diseased sheep may not be due to unsatisfying sensitivity of the new assay, but appears to reflect the absence of antibodies in response against BDV. Thus,

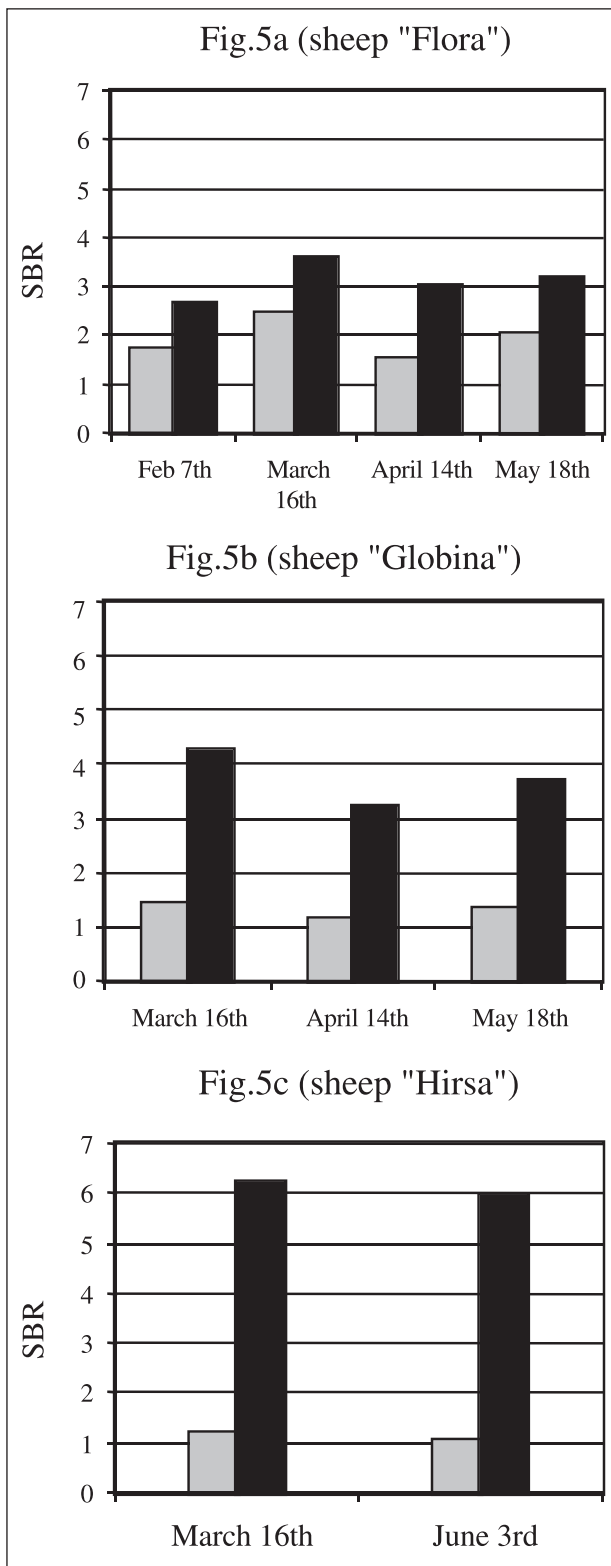


Figure 5: Time course study of antibody to p40 and p24. Three individual sheep (Fig. 5a, b and c) were observed during 4 months and tested repeatedly for the presence of antibody to BDV p40 and p24. SBR of p40 (gray columns) and SBR of p24 (black columns) are plotted against time.

serological analysis of serum antibodies as a diagnostic tool to detect individual sheep naturally infected with BDV is of limited value. However, epidemiological studies using large numbers of sera are still valuable.

In our study we considered 6% of sheep sera from the endemic area and 2% from the non-endemic area to react specifically to BDV antigen. Although only 2% of sera from the non-endemic area were positive, the difference between the endemic and the non-endemic group is statistically not significant. The data compare favorable to other studies from endemic regions. In Bavaria, a seroprevalence of 0.4% was described among 978 sheep using the complementation-fixation test (Wagner, 1970). In Switzerland, one of 283 sera from slaughtered sheep (0.35%) was positive by indirect immunofluorescence assay (Rohner-Cotti, 1992). Among 666 sera collected randomly in Eastern Germany 4% were positive using the same assay (Dürwald, 1993). Differences in the number of positive sheep may be explained by the fact, that these studies were carried out in different geographical regions within a time frame of three decades and that different serological assays were used. In contrast to the relatively low number of seropositive sheep we found during our study, a recent publication claims to detect up to 33% seropositive sheep (Hagiwara et al., 1997); another one describes a population of horses with up to 41% seropositive individuals (Inoue et al., 2002). Taking into account that the majority of serological assays have been optimized for highest sensitivity rather than specificity (Staheli et al., 2000) and that serological assays used by different bornavirus laboratories in Germany varied considerably with respect to sensitivity and specificity, the importance of some of these studies has to be questioned. Furthermore, the affinity as well as the avidity of the detected BDV-specific antibodies has to be further investigated before final conclusions can be made.

Because p40 was shown to be the main target antigen for BDV-specific T cells (Hausmann et al., 1999; Planz and Stitz, 1999), we used p40 for DTH reaction and an *ex vivo* interferon gamma assay. None of the healthy sheep tested was positive in either assay. Unfortunately, no BDV-diseased sheep or horses were available during the course of this study. Therefore, it remains to be shown, whether naturally diseased sheep or horses also test negative in those assays. Interestingly, DTH reactions performed on experimentally infected rats were negative (J.A. Richt, personal communication). It is possible that virus-specific T cells are mainly restricted to the brain and that the number of these cells in the periphery is too low to trigger a DTH reaction (J.A. Richt, personal communication). Hence, DTH reaction or interferon gamma assay did not provide evidence of additional sheep infected with BDV in endemic areas that were missed by our serological analysis. Therefore, ELISA is preferred to DTH to find healthy BDV-infected individuals as shown in this paper for sheep or suggested previously for rats (J.A. Richt, personal communication).

With 2% of sheep sera from the non-endemic area calculated to be positive for BDV-specific antibodies, the question arose, whether these antibodies indicated previous exposure to BDV. So far no case of BD has been described in any species from this non-endemic area of Switzerland in the past or during a recent intensive analysis of several hundred brains from sheep, goats and cows as part of a survey to determine the presence of transmissible bovine spongiform encephalopathy (Prof. Vandeveld, University of Berne, Switzerland, personal communication). Therefore, we assume that even rare cases of BD in this non-endemic area of Switzerland would have been detected. We thus conclude that antibodies to BDV antigen from sheep in non-endemic areas were not induced by this virus but instead by a crossreactive antigen. In a recently published study 33% of sheep living on the island of Hokkaido in Japan were found to have antibody to p24, p40 or both antigens (Hagiwara et al., 1997). Interestingly, in this area classical BD has never been diagnosed in sheep. Therefore, it might be possible that related viruses are present in regions outside of areas where BD is diagnosed (Lundgren et al., 1995; Nowotny and Weissenböck, 1995) (Nowotny et al., 2000). In the future, reliable serological techniques may prove to be beneficial to find new subtypes of BDV and similar studies in other regions may change our understanding on the geographical distribution of this virus family.

Three sheep with a SBR higher than 3.6 for p24 were found in a flock from the endemic area (Fig. 3, 5). These animals belonged to the same flock in which one sheep had died of BD one year ago. Samples from the three sheep were monitored serologically between February and June 2000. During this time SBR calculated for p40 or p24 remained similar but the sheep stayed healthy and no sign of BD was evident (Fig. 5). These three sheep may have overcome a subclinical infection with BDV with or without elimination of the virus. The possibility that survivors can successfully eliminate the virus is supported by a previous report (Rohner-Cotti, 1992).

Interestingly, the three sheep that we analyzed over several months had detectable amounts of specific antibodies only to p24 but not to p40. In sera of other sheep antibody to p40 was prevalent. The relevance of this finding is unclear but has been noted in a previous study (Hagiwara et al., 1997). A recent study showed that experimentally infected cats reacted mainly against p40, whereas naturally infected cats had a more p24-directed immune response (Johansson et al., 2002). Furthermore, cats experimentally infected with BDV strain V, one of the laboratory strains originating from a horse, showed a more p40-directed immune response compared to cats experimentally infected with the feline variant of BDV. The authors assume that cats may be able to suppress an acute infection with the feline variant of BDV and develop a persistent infection leading to a strong p24-response (Johansson et al., 2002). Whether the predominance of p24-specific or p40-specific antibodies in sheep could possibly be indicating the existence of different viral strains has to be further elucidated.

Our newly developed ELISA system could provide the tools to further investigate the occurrence and possible disappearance of antibodies against p24, p40 or other viral antigens from sheep of endemic and non-endemic areas. Antibody production to viral proteins might be influenced by particular virus isolates in certain areas or by cofactors postulated in endemic areas that may lead to BD. The work of Nowotny and coworkers (Nowotny et al., 2000) showed that infections with Borna viruses might be missed by PCR analysis. Therefore, serological screening of a large number of animals using an ELISA can be a valuable tool to acquire more information on the distribution of Borna viruses in endemic as well as in non-endemic areas.

As the possible zoonotic potential of BDV or Borna related viruses is still a matter of debate, further investigations in the epidemiology of BD in the endemic and the non-endemic areas appear relevant.

La réponse immunitaire humorale et cellulaire du mouton face au virus de la maladie de Borna dans des zones endémiques et non-endémiques

La maladie de Borna (BD, Borna Disease) est une maladie presque toujours mortelle des moutons et des chevaux, endémique dans des zones restreintes d'Europe occidentale. Il est intéressant de constater que des anticorps contre le virus de la maladie de Borna (BDV, Borna disease virus) ont été trouvés sur des moutons provenant aussi bien de zones d'endémiques que de zones non endémiques. Dans l'étude présentée, les réactions des moutons face à la maladie de Borna ont été examinées dans des régions endémiques et non endémiques géographiquement comparables. Des anticorps contre l'antigène BDV n'ont pu être mis en évidence par ELISA et par immunofluorescence indirecte (IFA) que chez 3 des 6 moutons présentant une maladie de Borna vérifiée pathologiquement. Un sérum a réagi positivement à l'IFA mais pas à l'ELISA alors que deux autres sont restés négatifs dans les deux tests, ce qui démontre que tous les animaux atteints ne développent pas des anticorps spécifiques BDV. 6% des moutons sains provenant de la zone d'endémie (6/106) et 2% des moutons provenant de la zone non endémique (4 sur 192) présentaient des anticorps sériques, dirigés soit contre le BDV p40 ou p24. Aucun des animaux examinés ne présentait une réponse immunitaire cellulaire contre le BDV p40. Chez certains animaux sains provenant de la zone d'endémiques, le taux d'anticorps sériques contre le BDV p24 est resté élevé durant plusieurs mois sans que ces animaux aient présenté de symptomatologie clinique. Il est possible que des infections se produisent soit par le BDV soit par des virus apparentés avec une fréquence basse chez des moutons dans les zones non endémiques, ce qui conduit à la production d'anticorps contre l'antigène BDV spécifique. En outre, des différences entre les souches virales ou des facteurs environnementaux peuvent influencer le tableau clinique.

Reazione immunitaria sierologica e cellulare delle pecore contro il virus della malattia di Borna in aree endemiche e non endemiche

La malattia di Borna (BD, Borna Disease), endemica in una zona ristretta del centro Europa, è una malattia delle pecore e dei cavalli prevalentemente ad esito letale. Di vasto interesse è la scoperta nelle pecore, di un anticorpo contro il virus della malattia di Borna (BDV, Borna disease virus) ritrovato sia in zone endemiche sia non endemiche. Nello studio qui presentato viene analizzata la reazione sierologica delle pecore contro la BD in aree endemiche e non endemiche geograficamente simili in Svizzera. Anticorpi contro l'antigene BDV sono stati trovati tramite ELISA e immunofluorescenza indiretta (IFA) solo in tre delle sei pecore colpite dalla BD. Un siero ha reagito positivamente all'IFA ma non con ELISA, mentre altri due sieri erano negativi in entrambi i test, ciò che significa che non tutti gli animali malati di BDV producono anticorpi specifici. Con ELISA il 6% degli animali sani esaminati clinicamente (6/106) provenienti dalla zona endemica e il 2% dalla zona non endemica (4/192) mostravano anticorpi nel siero che erano diretti o verso BDV p40 o p24. Nessun animale esaminato mostrava una risposta immunitaria verso il BDV p40. In alcuni animali sani delle regioni endemiche la concentrazione di anticorpi nel siero rimane alta durante vari mesi, senza che questi animali mostrino una sintomatologia clinica. Probabilmente le infezioni di BDV o con virus apparentati compaiono con una frequenza inferiore nelle pecore di aree non endemiche la qual cosa provoca una produzione di anticorpi verso l'antigene specifico del BDV. Inoltre è stato possibile definire le differenze nel quadro clinico tra ceppi virali o fattori ambientali.

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