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THE IMMUNOPATHOGENESIS OF BORNA DISEASE VIRUS INFECTION

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1. ABSTRACT

Borna disease virus (BDV) infection represents an excellent model system to study immunopathological mechanisms based on a T cell-mediated immune reaction in the central nervous system. The single-stranded RNA Borna disease virus, a member of *Bornaviridae* in the order of *Mononegavirale*, lacks cytopathogenicity both *in vitro* and *in vivo*. After experimental infection BDV causes a persistent infection of the central nervous system and induces Borna disease, an immune-mediated encephalomyelitis. The infiltrating immune cells have been characterized as CD4-positive, CD8-positive T-cells, macrophages and B cells. CD8-positive T cells represent the effector cell population exhibiting antigen specificity for the nucleoprotein.

2. INTRODUCTION

The first descriptions of a disease characterized by central nervous symptoms in horses can be found in the veterinary literature at the end of the 18th century and even more sophisticated reports on a "disease of the head" (hitzige Kopfkrankheit), brain fever, subacute meningitis, or hypersomnia of horses" at the beginning of the 19th century (1). The disease attained more attention in 1895 when almost all horses of a cavalry regiment in the town of Borna in Saxony near Leipzig succumbed to an epidemic with severe central nervous system symptoms. After this time point the name Borna Diseas (BD) was adopted. Although investigations on the disease caused by the Borna Disease Virus (BDV) had already been undertaken in the 1920's, the nature of this virus had remained obscure for a long time. Only recently, knowledge on the type of nucleic acid, the organization of the genome, the characterization of virus-coded proteins and the classification of the virus have increased our understanding of the agent causing a neurological disease. In this review the properties of the virus and the biological properties of the respective host will be discussed, since they determine the role of the virus being a pathogen for animals and possibly for man.

The virus etiology of BD had already been established as early as 1925, when Zwick and Seifried (2) proved it´s transmissibility. They were then able to fulfil Koch's postulates by transmitting the disease from horses to rabbits and back to horses. These initial studies, which had been proceeded by only a single publication in 1924 (2), led to an intensive investigation of this central nervous system (CNS) disease by Zwick and his associates resulting in several publications on the biological, physical and chemical properties of the virus.

The BD has been investigated during the past 70 years, because of it´s relevance for veterinary science; it is an important model for virus-induced CNS disease and it has been shown to be useful for the investigation of immunolopathological mechanisms. Today, BD has been observed in a variety of animal species including cats, dogs, cattle, sheep and horses. In addition, the potential

role of BDV as a pathogen in human psychiatric diseases has increased interest in the investigation of this virus and the pathogenetic pathways. Early experiments using radioactive labelled nucleosides (3), or inhibitors of nucleic acid synthesis, had suggested that BDV is an RNA virus (4). Molecular biological analysis revealed important data on the type of the genomic nucleic acid, the genomic organization and the synthesis of BDV-specific proteins. This work became feasible by the establishment of BDV-specific cDNA clones (5, 7). Those data were achieved by substracting expression libraries derived from non-infected and from BDV-infected rat brains (5), or from non-infected and BDV-infected MDCK cells (6). The different libraries were either screened with cDNA probes prepared from BDV-infected rat brain RNA enriched for BDV-specific sequences by substraction with normal rat brain cDNA on the level of nucleic acids (5), or by the use of BDV-specific monoclonal antibodies (Mab) on the level of protein synthesis (6). Today, it is commonly accepted that BDV is a non-cytolytic single-stranded RNA virus, and represents the only member of *Bornaviridae* in the order of *Mononegavirales*. BDV is highly neurotropic and cell-associated. The 8.9 kb size genome with negative polarity is replicated in the nucleus. Little is known on the consecutive steps involved in virus replication. There is some evidence that the major glycoprotein (gp94) mediates penetration by membrane fusion after the fusion peptide becomes exposed by proteolytic cleavage of the glycoprotein. The cellular splicing machinery is required to process some of the primary RNA transcripts. A variety of mechanisms are used to regulate gene expression, including the use of overlapping reading frames, overlapping transcription units, alternate RNA splicing and leaking scanning of ribosomes during protein translation. The replication cycle seems to be completed within 24 hrs. If at all, only little infectious virus is released from infected cells. The genome encodes for at least 6 different known viral proteins. The first open reading frame (ORF I), which is the prominent 3'-ORF, encodes the 38/39 kDa protein (also known as p40), the putative nucleoprotein (NP). Recent investigations have suggested that NP has two functions, nuclear localisation and export activity. Furthermore, it apparently plays a major role in the nucleoplasmic transport of BDV-ribonucleoprotein complex (RNP; ref. 8). ORF II codes for a 24 kDa protein (also known as p24), representing the putative phosphoprotein (P). This protein is phosphorylated in its serine residues suggesting that the function of this protein is controlled by cellular kinases (9). The products of ORF I and ORF II (p40 and p24) have been shown to form a complex which has been deduced from coprecipitation by monoclonal antibodies as well as from one-dimensional peptide digestion. Recently, an additional open reading frame (ORF X) has been identified, greatly overlapping with ORF II, encoding a nonglycosylated BDV protein designated p10. This protein binds directly to the phosphoprotein and indirectly to the nucleoprotein. In this respect it was shown, that the interaction of p10 with the phosphoprotein is important for the association with BDV-specific intranuclear clusters that may represent the sites of virus replication and transcription (10-13). Furthermore, it was shown, that N and P protein but not p10 carries nuclear localisation signals (NLS; ref. 14). The third ORF encodes the p16 protein which was described as glycosylated, resulting in gp18 (also known as the 14 kDa protein), and represents the putative matrix protein (M). The ORF IV encodes p57 that is found in its N-glycosylated form at approx. 94 kDa (described as gp94) and represents the putative precursor glycoprotein of the virus (GP); cleavage products of this GP have been reported to result in molecular masses of approx. 43 kD by the subtilisin-like cellular protease furin (10a). Finally, the gene product of ORF V, which is localized at the 5'-end of the viral antigenome, has been identified so far as a 180 – 190 kD protein, representing the RNA-polymerase of BDV. A recent report by Walker et al. shows that the L-polymerase of BDV is also phosphorylated, making this protein a further candidate for BDV-host cell interactions (15).

The cellular factors and processes crucial for BDV replication are poorly defined. Recently it was shown that the Raf/MEK/ERK signaling pathway is activated upon BDV infection after acute infection. Already one hour after infection ERK activation is detected. This very early time point at which ERK activation is detected correlates well with the requirements of the pathway early during infection; however, it would suggest that gene expression is not involved in activation of the kinase cascade. In addition, it was shown that ERK is activated in different persistent BDV-infected cell lines to distinct levels (16). Furthermore, BDV caused constitutive activation of the ERK1/2 pathway and activated ERKs were not translocated to the nucleus efficiently in persistently infected PC12 cells. That might account for the absence of neuronal differentiation of BDV-infected PC12 cells treated with nerve growth factor (NGF; ref. 17).

Inhibition of the cascade by the MEK inhibitor U0126 resulted in a block of BDV spread in cell culture which reduces virus yields up to 99%. Inhibition was observed in two different cell lines highly susceptible to BDV infection, showing that the effect is not restricted to a particular cell type (16). In addition to the function of a MEK-inhibitor as an antiviral, so far, amantadine and ribavirin were described as anti-BDV drugs. The effect of amantadine is controversally discussed and ribavirin only reduces infectivity in vitro by one $\log_{10}$ (18-23).

3. DISSEMINATION OF BORNA DISEASE VIRUS

After experimental infection of adult rats by various routes, including intracerebral, intraocular, intranasal and intramuscular injection, infectious virus and virus-specific antigen can be detected in high concentrations in the cerebrospinal fluid, brain, retina, , peripheral nerves and adrenal gland (24-28). After experimental intranasal infection, virus-specific antigen can be identified in the neuroreceptors in the olfactory epithelium and consecutively migrates to the brain (28). *In vivo*, the virus replicates preferentially in cells derived from the neural crest such as neurons, astrocytes, oligodendrocytes and ependymal cells without exhibiting signs of direct cytopathogenicity (25, 26, 29-31). *In vitro*, in addition to cells derived from brain tissue, other cell ty-

pes such as skin, kidney and testis, can be infected after incubation with clarified homogenates from infectious rat brain (32-35) or after cocultivation (33). The virus is tightly cell associated and also lacks apparent cytopathogenicity *in vitro* (33).

In infected rats, which usually survive the acute infection and develop chronic disease, virus antigen can be found in cells of the peripheral nervous system such as the Schwann cells (25), and the virus can be located by culture and/or reverse transcriptase polymerase chain reaction (RT-PCR) in extraneural tissues (36, 37), including the peripheral blood mononuclear cells (38). From many other tissues and organs conflicting data have been reported, particularly in the older literature. In the most recent study however, investigating the presence of infectivity by virus titration on BDV-susceptible indicator cells and of the presence of BDV-specific antigen by immunohistological methods clearly revealed the absence of the virus from non-neural tissue (39). These results have been recently confirmed employing both immunocytochemical methods using BDV-specific mAb and by *in situ* hybridization (40). However, the previous findings have been extended in so far as in BDV-infected adult rats, in which infectious BDV could only be demonstrated in the CNS, virus specific antigen and viral nucleic acid was found in peripheral nerves, e.g. within muscles and in the axons of the sciatic nerve, a finding that stresses the importance of the spread of the virus via nerves, especially the axonal transport of BDV (27, 28, 41). Furthermore, virus-specific antigen and the genome was detected in the Auerbach's Plexus and the Plexus mesentericus in the intestines. In all cases of the presence of BDV in peripheral nerves of rats infected as adults, no virus was found in organ-specific cells, but was strictly restricted to nervous structures which stands in contrast to observations made in rats infected with BDV as adults and immunosuppressed by Cyclosporine A (CSA; refs. 40 & 42).

In the rat, virus-specific protein was demonstrated in neuro-receptor cells of the olfactory epithelium in intranasally infected animals (28). Since the immunohistological demonstration was possible as early as six days and as late as 26 days post infection (p.i.) those cells have to be regarded as susceptible for infection and virus replication.

In a study already published in 1963 (ref. 43), a most important finding was described that was only about 20 years later reinvestigated and finally understood. Nitzschke (43) had described the presence of infectious virus in various organs, including spleen, liver, kidney, and even in the blood of rats infected as newborns within the first two days of life. Later work done in newborn infected (40, 44), and in immunologically impaired adult rats (42), revealed the presence of virus and virus-specific antigen in various organs. In a most recent study, employing infectivity assays as well as immunohistology and *in situ* hybridization, the presence of BDV was demonstrated in all organs tested from newborn infected and immunocompromised rats (40). Interestingly, the virus was not only associated with peripheral nerves and nerve fibers but could be demonstrated in organs-specific cells (40, 42). In immunocompetent infected rats, BDV RNA has been demonstrated in blood in the late chronic phase of the disease (45, 46). Finally, and most convincingly, it has been demonstrated that newborn infected rats which chronically excrete the virus e.g. via tears, saliva and urine, can infect other rats having close contact (28, 40, 43, 47). Molecular studies also reported on the presence of BDV RNA in conjunctival fluid, saliva and nasal secretions of seropositive animals. These findings are in good agreement with the interpretation that natural infection most probably occurs via excretions and the olfactory route (28, 36, 47); however, oral infection via the recurrent pharangeal nerve cannot be excluded (36). The direct access of virus to the CNS via the nerve endings, especially olfactory receptor neurons (48) has been demonstrated for various other viruses such as the neurotropic influenza virus (49), herpes simplex virus (50) and the Semliki Forest virus (51).

After infection, the virus replicates rather quickly in cells of the CNS, although the development of clinical symptoms usually requires longer time. Already four to six days after infection virus-specific proteins as demonstrated by Western Blot analysis can be found. Likewise, BDV-specific proteins are detectable by immunocytochemistry as early as four days in single ependymal cells of the lateral ventricles after intracranial (i.c.) infection. These cells show a distinct granular staining in the nucleus but also the cytoplasma of single cells is stained. Six days p.i., a few neurons of the hippocampus (layers CA3/CA4) and of the frontal cortex contain virus specific proteins. After eight days, the number of cells with BDV-antigen significantly increases. Especially large numbers of neurons in the CA3 region harbor virus-specific proteins. Positive cells are also found in the diencephalon and amygdala. At this time point a fine granular staining of the cellular extensions of neurons can be observed additionally to the strong nuclear and pericaryc reaction. Finally, at day 10 p.i., BDV can be demonstrated in all cortical and brain stem areas. The intensity of the staining reaction increases until day 22 p.i., particularly of the neuropil in the stratum oriens and stratum radiatum of the CA3/CA4 regions of the hippocampus. Thereafter, the intensity of the staining reaction decreases and beyond day 70 p.i., BDV is essentially found in the hilus area of the hippocampus, the septal nuclei and in periventricular regions. During the later stages of the infection the cytoplasmic staining decreases and the intranuclear reaction predominates again in almost all cells. The types of cells containing BDV-specific proteins as far as they have been identified comprise neurons, ependymal cells, astrocytes and oligodendrocytes, whereas endothelial cells of brain blood vessels have been found negative (26, 52).

4. DISSEMINATION OF IMMUNE CELLS IN BORNA DISEASE

The presence of cellular infiltrates in the brains of BDV-infected animals represent the corner stone for studies into the cellular immune response after BDV-infection and the consequences of this response for the outcome of the infection in particular. Although

perivascular and parenchymal infiltrates had been observed upon histological examination, their importance remained unclear until the publication by Narayan et al. in 1983 (ref. 24). In this paper not only the histological alteration in the brain were scrutinized, but they were also related to the appearance of clinical signs and therefore was the starting point on investigations on the pathogenesis of this virus-induced encephalopathy. In the rat, the encephalitic reaction was carefully analyzed and perivascular infiltrates were characterized by immunohistological methods (26, 53, 54). These investigations revealed the phenotype of cells present in the encephalitic lesions by use of mAb directed against several surface markers of T lymphocytes, B lymphocytes and macrophages. It was shown that cells reacting with a mAb against CD3, a common T cell marker, were represented by lymphatic cells with round nuclei, with dense heterochromatin and scanty cytoplasm. These cells attained about 50% of all cells present in perivascular infiltrates at day 12 p.i., and culminated between days 22 and 30 p.i. Beyond this time, cells carrying the CD3 marker slightly decreased again, reaching again a value of 30% at day 70 p.i.

The population of T cells was further divided into cells CD4-positive Helper and CD8-positive cytotoxic T cells. Antibody used to detect CD4-positive cells again only stained small cells with round nuclei, dense heterochromatin and scanty cytoplasm. The CD4-positive cells represented the most frequent type of T cells in the perivascular encephalitic lesions. At day 12 p.i., they made up 15% of all perivascular cuffs, and thereafter their number increased to about 29% at day 22 p.i. Beyond day 30, a decline of cells with the helper phenotype was established and a level of 13% was reached at day 70 p.i. Single CD4-positive T cells were found in the brain parenchyma but they clearly predominanted in the perivascular cuffs. Cytotoxic T cells were recognized by the same morphological characteristics mentioned above. These cells were found also in the perivascular cuffs, but predominantly in the brain parenchyma and attained about 10% of all cells (26). In another investigation, cells carrying this marker reached up to 20% of the cells present in the encephalitic lesions (55). However, according to both reports, CD8-positive T cells are represented in lower numbers, when compared to CD4-positive T cells. Cells reacting with the ED1 mAb, specific to a marker on macrophages, represented the majority of inflammatory cells in the perivascular and parenchymal infiltrates, during the entire investigation period. They were recognized by their morphology as large cells with ovoid to kidney-shaped cytoplasm.

A vigorous humoral immune response can be seen in BDV-infected and otherwise untreated animals of various species. However, for the current knowledge, the presence of virus-specific antibodies is not related to disease. The detection of BDV-specific antibodies without disease symptoms also proves the existence of an inapparent infection, which has been reported for horses and sheep (56). Alternatively, the presence of antibodies could reflect the presence of the virus in the preclinical stage, i.e. during the variably long incubation period before clinical symptoms can be seen. In the sera of experimentally infected rats, antibodies directed against the nucleoprotein (p40) and the phosphoprotein (p24) preponderate. In experimentally infected rats, antibodies against both major BDV-specific proteins are always generated; however, the amount of antibodies against phosphoprotein is sometimes lower, or the appearance is delayed. Specific antibodies to BDV can be detected in the sera of the infected rats nine to 12 days after infection. In the cerebrospinal fluid of infected animals, virus-specific antibodies are readily detected representing an oligoclonal immunoglobulin response (57). In rat brains, an abundant local IgG production with isotype variations has been demonstrated, and no damage of the blood brain barrier has been observed (26, 58). The same authors have forwarded the idea that locally produced anti-BDV antibodies may influence viral gene expression.

The presence of neutralizing antibodies in BDV-infected hosts was a matter of long and controversial discussions. Some investigators had reported on virus neutralizing activity in serum and cerebrospinal fluid (32, 40, 59-62), whereas others reported no evidence of neutralizing antibodies in any stage of the disease (27, 29 39). The final proof for the presence of neutralizing antibodies, at least in rats after experimentally BDV-infection, was provided when Mab could be generated which reacted with the major viral glycoprotein (gp94), and inhibited infection both *in vitro* and *in vivo* (46). Interestingly, it appears that neutralizing antibodies are only synthesized *in vivo* after the virus is present in the blood, which only occurs late after infection in the chronic phase of the disease, and provides an explanation for the delayed presence of neutralizing antibodies. Furthermore, when these Mab's were given to rats prior to BDV-infection, BD could be prevented.

5. IMMUNOPATHOLOGY OF BORNA DISEASE

The crucial importance of the immune system in BDV infection becomes obvious when athymic or immunocompromised animals are infected. Rats treated with cyclophosphamide or CSA and athymic rats do not show Borna disease or the acute inflammatory reaction (29, 39, 44, 63, 64). The first experiments that indicated the importance of the immune reaction for the pathogenesis of BD, however, came from earlier experiments in which rhesus monkeys had been used (65). It was then observed that monkeys which had undergone splenectomy prior to BDV-infection showed a different clinical and histopathological picture. Rhesus monkeys that had not been splenectomized developed a severe disease with neurological symptoms including paralysis, pareses and, in the late phase of the disease increasing apathy.

In a more detailed study the immunopathological basis of BD was established in rats (29). It was shown that immunosuppression with cyclophosphamide of BDV-infected rats resulted in the absence of encephalitis and disease, whereas these persistently infected tolerant rats were susceptible to the disease after adoptive transfer of immune spleen lymphocytes.

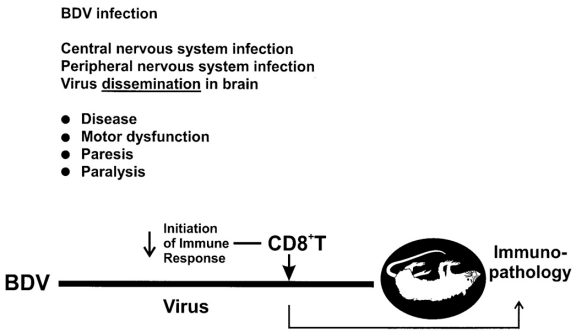

Figure 1. Immunopathology and Borna disease in rats.

Virus-specific nucleic acid and infectious virus, in addition to virus-specific antigen, were found in immunocompromised animals in amounts comparable to fully immunocompetent rats (27, 29, 39, 55, 64). Strikingly, immunocompromised rats, show no destruction of the retina, although the virus persists in the eye, and the rats do not become blind despite the presence of virus in retinal layers (29). The importance of the immune response for the pathogenesis was further stressed by showing that adoptive transfer of lymphocytes from BDV-immune rats into immunoincompetent animals resulted in full-blown BD (24, 63). In CSA-immunosuppressed rats, it was demonstrated by various approaches that no virus-specific T cells were present, and rats were protected from disease (63).

Numerous B cells can be detected in encephalitic lesions in the later stages of the acute disease. Athough antiviral antibodies including neutralizing antibodies can be found in the serum of infected rats (24, 46, 59, 62), several lines of evidence indicate that antiviral antibodies do not play a significant role in the immunopathogenesis of BD (24, 39, 66). Experiments with the immunosuppressive CSA supply an additional argument against the involvement of antibodies in the immunopathogenesis of BD (63). Interestingly, treatment of BDV infected rats with suboptimal doses results in the development of an encephalitis in the absence of an anti-BDV antibody response. Additionally, while an intracerebral challenge of CSA-treated rats with BDV does not result in immunopathological disease, it does induce a B cell response (63). These facts together demonstrate that BD is due to a virus-induced immunopathological reaction at the T cell level. Because restriction elements are most important in T cell-mediated immune phenomena, the presence of the major histocompatibility complex (MHC) class I and II antigen was scrutinized in the brains of the BDV-infected rats. These self antigens are detected on various cell types upon immunohistological characterization, namely perivascularly but also on oligodendrocytes, microglial and ependymal cells (26, 31, 53, 64). It is of note that MHC class II antigen was also detected in areas where no inflammatory reactions took place, arguing for a general induction and expression of MHC class II in the brain of BDV-infected rats.

By characterizing the brain cells that express MHC class I antigen, it became evident that this self antigen could be demonstrated on neurons and astrocytes *in vivo* and *in vitro* (31, 54, 67). Furthermore, MHC class I expression was distributed throughout the brain tissue early after infection, whereas MHC class II was restricted to perivascular localizations at this time point. The pathways of immunopathology in Borna disease are summarized in figures 1-3.

5.1. Evidence for the role of $CD8^+$ T cells

First support for the importance of CD8-positive T cells was obtained when BDV-infected rats were treated with transforming growth factor (TGF-beta2; ref. 55). TGF's act as multifunctional cytokines with potent inhibitory activity on growth, differentiation and effector functions of activated T and B lymphocytes as well as macrophages (reviewed in refs. 68 & 69). Treatment of BDV-infected rats with TGF-beta2 revealed a transient reduction in the severity of clinical symptoms that was paralleled by a significant reduction of the inflammatory reaction in the brain. Thus, TGF-beta negatively interfered with the development of T cell-mediated disease if given systemically (55, 70). Immunohistological investigations revealed slightly reduced CD4-positive T cell numbers and no changes in macrophage counts in encephalitic lesions of TGF-treated rats, whereas CD8-positive T cells were virtually absent from the perivascular inflammatory reaction at early time points after infection and treatment. However, and most interestingly, the appearance of CD8-positive T cells late after infection and treatment was directly correlated with the onset and severity of clinical symptoms. In addition, the expression of MHC class II antigen was found to be significantly reduced in the brain of TGF-treated Borna disease virus-infected rats, whereas MHC class I expression was not. Since CD8-positive T cells are potent producers of immune interferon (IFN), and since IFN-gamma also regulates MHC class I and class II expression (71, 72), the absence of CD8-positive T cells in the brain of TGF-treated rats might be responsible for the observed reduction of MHC class II antigen (55). This interpretation agrees with the finding that BDV-infected astrocytes produce *in vitro* IFN alpha/beta (35) that was previously described as astrocyte-IFN (73). The type I IFN's: IFN?alpha/beta and, in particular, IFN produced by astrocytes, up-regulate MHC class I but not class II expression (73, 74). This finding provides a good explanation for the IFN-gamma independent presence of MHC class I in TGF-treated rats. In summary, in spite of the presence of CD4-positive T cells, the absence of CD8-positive T cells and reduced expression of the restriction elements for cell-mediated immune responses led to an initial inhibition of the encephalitic reaction and clinical symptoms.

Thymectomized rats that were treated with antibodies to deplete all T cells, or the CD8-positive T cell compartment selectively, showed decrease or even prevention of the local inflammatory reaction (75). Although antibodies directed against CD4-positive T cells were also found to influence the disease to some extent, they had to be given at high doses and for a prolonged time to exert a beneficial effect. Rats that had not undergone thymectomy prior to infection, but were treated with anti-CD8 antibodies showed a considerable delay in the onset of

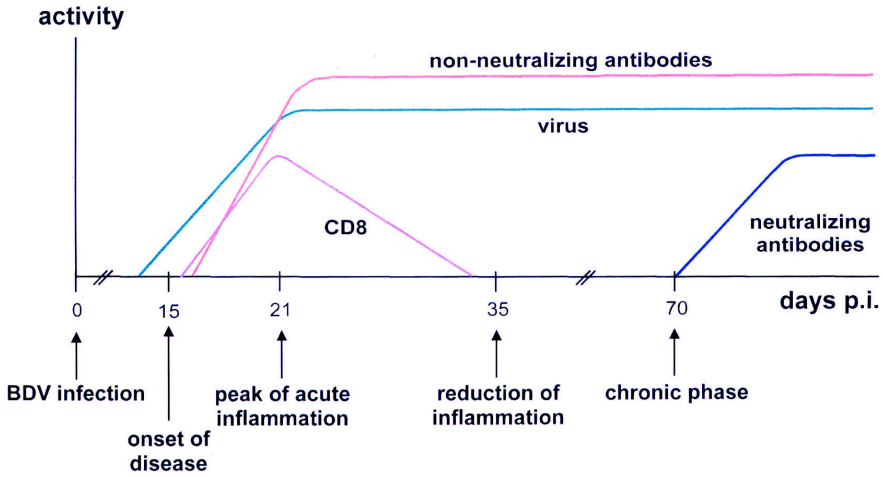

Figure 2. Kinetics of immune mechanisms in Borna disease.

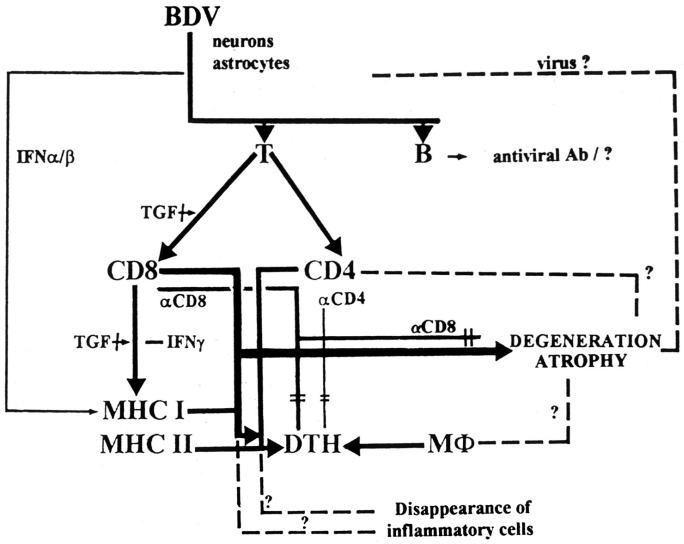

Figure 3. Mechanisms of immunopathology in Borna disease.

disease. In all experiments, CD8-specific Mab appeared to be much more effective, easily preventing the immunopathological disease, whereas antibodies directed against CD4-positive cells were significantly less effective. These observations support the concept that CD8-positive T cells play an important role in the pathogenesis of BD. Therefore, a sequential role of an initial CD8-positive T cell response was proposed that is decisive in triggering the local CD4-positive T cell-mediated delayed type hypersensitivity reaction in the brain after BDV infection (75). Likewise, treatment begun after infection resulted in some delay of the inflammatory reaction, and consequently of BD, but did not affect the severity of encephalitis or long-term disease. This very limited effect of monoclonal antibody treatment late after infection further suggests an effective and rapid activation of T cells in the periphery of BDV-infected rats (63). This might be due to the known fact that after intracerebral infection, which is the usual route used in experimental infection in rats, considerable amounts of virus gain access to the periphery (76). However, in the case of intranasal experimental infection, the same argument might be used, since dendritic cells and macrophages act as antigen-processing and -presenting cells at the site of inoculation.

5.2. Role of $CD4^+$ T cells in Borna disease virus immunopathogenesis

The importance of T cell subsets in the pathogenesis of BD became obvious when homogeneous virus-specific T cell lines were established. Lymphocytes were obtained from regional lymph nodes of rats that had been immunized with the nucleoprotein p40 purified virus-specific antigen and were cultured and restimulated *in vitro* (53, 77). Analysis of this cell line revealed BDV-specificity, MHC class II restriction and the phenotypical markers of CD4-positive helper/inflammatory cells (77). Passive transfer of this cell line into BDV-infected cyclophosphamide immunosuppressed healthy recipients resulted in severe disease and death as early as day 5 after the injection of effector cells (53, 77). In contrast, passive transfer into uninfected rats did not result in encephalitis or disease, demonstrating that this BDV-specific T cell line,

by itself, was not encephalitogenic. These results, together with the immunohistological characterization of inflammatory cells in the brain of BDV-infected rats, and data obtained with other T cell lines (66), strongly suggested that BD is caused by a delayed type hypersensitivity reaction (DTH).

The importance of MHC class II-bearing cells in the pathogenic mechanism has not yet been determined, especially since it has not yet been possible to demonstrate their role in antigen presentation of BDV-specific antigen in the brain. However, some evidence has accumulated that provides better insight into the mechanisms of pathogenicity and the T cell subsets involved. In order to define a cell type in the CNS which might be relevant to the *in vivo* situation in BD, possible functional interactions between CD4-positive BDV-specific T cells and astrocytes were tested *in vitro*. Since astrocytes are target cells of BDV infection in the rat brains (25, 26, 67, 78), and they are potent antigen presenting cells, they may play a role in the immunopathology after viral infections, or in autoimmune disorders in the CNS (71, 79), it would be of interest to investigate T cell-astrocyte interactions. With regard to MHC class II expression on astrocytes, it was shown that the elevated expression of this self antigen, induced by IFN-gamma, increased the proliferative capacity of the BDV-specific CD4-positive T cell line *in vitro* (53). In the brain of infected rats (55) and in astrocytic cultures *in vitro* (34, 35, 53), the virus alone is not able to induce the expression of MHC class II.

Interestingly, and relevant to the *in vivo* situation, BDV-infected astrocytes retained their full ability to present BDV-specific antigen, a fact that again proves that BDV infection does not essentially interfere with vital cell functions (34). Furthermore, the expression of MHC class II on BDV-infected astrocytes was a prerequisite to serve as target cells for *in vitro* cytotoxicity by the CD4-positive T cell line (34). This BDV-specific CD4-positive T cell was able to lyse syngeneic, IFN-gamma-treated, persistently infected astrocytes, and lysis was significantly reduced by antibodies directed against MHC class II antigens. It should be kept in mind, however, that CD4-positive T cells might acquire cytotoxic activity upon *in vitro* cultivation as it has previously been demonstrated (80, 81). Furthermore, it has been shown in other models that CD4-positive T cells are more aggressive after *in vitro* cultivation, and may not reflect the entire reaction of the immune system during a natural infection. Pathological alterations can be induced by CD4-positive T cell clones, in the absence of CD8-positive T cells, although this reaction has been shown to be dependent on CD8-positive T cells *in vivo* (82). In addition, CD4-positive T cells might be responsible for inflammatory reactions that, however, do not cause disease, which is only seen after CD8-positive T cells enter the site of inflammation (83, 84). It cannot be excluded that the BDV-specific CD4-positive cytotoxic T cell mentioned above, is rather exceptional or an *in vitro* artefact, since other CD4-positive T cells established with specificity for either the nucleoprotein p40 or phosphoprotein p24 protein were shown to lack cytotoxic activity *in vitro* (66). Here, CD4-positive T cell lines, specific for the unglycosylated 24 kDa antigen, have been established *in vitro*. In addition to phenotypic markers, these T cell lines and derived clones were shown to carry the alpha/beta T-cell receptor (TCR) and the beta-4 integrin (VLA-4). The latter has been shown to be the crucial molecule to allow entry of the activated cells into the brain after binding to VCAM-1 on endothelial cells (85-87). The concept has been proven valid also for BDV infection, when anti-alpha$_4$ integrin blocked the interaction of T cells with the endothelium of the blood-brain barrier, resulting in an inhibition of the disease process (88). An adoptive transfer of these CD4-positive T cell lines, which were characterized by their cytokine patterns as of the T helper1 or T helper intermediate type, resulted in neurological disease that was comparable to BD induced by intracerebral or intranasal infection (66). This observation clearly shows that cytotoxicity by CD4-positive T cells is not needed to induce the disease and that the rather unusual outcome of BD, namely rapid death, after the transfer of a CD4-positive T cell with cytotoxicity, can be regarded as being dependent upon this particular T cell line (47). Therefore, CD4-positive T cells do not appear to play a relevant role as effector cells via cell lysis in the pathogenesis of BD or, alternatively, act via the production of a cytokine, e.g. IFN-gamma which has various effects during an ongoing antiviral immune response.

Further insight into the immunopathological mechanisms was obtained when virus-specific CD4-positive T cell lines were adoptively transferred into recipients that had been rendered unresponsive by various methods. Adoptive transfers of helper T cell lines into cyclophosphamide-immunosuppressed BDV-infected recipients resulted in an increase of antibody titers and severe encephalitis associated with BD-specific neurological symptoms within two to three weeks after transfer. Interestingly, immunohistological analysis of the brains of rats displaying symptoms of Borna disease uniformly revealed the presence of CD8-positive T-cells in encephalitic lesions, in addition to CD4-positive cells that were found in the brain of recipients of the virus-specific CD4-positive T-cell line irrespective of whether neurological symptoms developed or not. However, recipient rats treated with antibodies against CD8-positive T-cells neither developed encephalitis nor disease symptoms (66). Therefore, CD4-positive T-cells appear to accumulate in the brain, cause perivascular inflammatory lesions but alone obviously do not cause disease. In contrast, the presence of CD8-positive cells apparently directly correlates with the development of neurological symptoms. The absence of neurological disease in spite of the presence of CD4-positive T cells and macrophages is very reminiscent of the situation described for TGF-treated rats (55). In addition, histological examination of brains from rats that had been successfully treated with anti-CD8 antibodies revealed slight to moderate encephalitic inflammatory reactions. Results from immunohistochemical analysis of these encephalitic reactions in healthy infected rats again stressed the importance of CD8-positive T cells in the pathogenesis, since the inflammatory cells comprise exclusively CD4-positive T cells and macrophages but no CD8-positive T cells (54, 75). We propose that the majority of the CD4-

positive T cells in the brain may not be antigen-specific, but are activated by an antigen-specific CD8-positive T cell response, or by the few antigen-specific CD4-positive T cells that produce relevant cytokines. It has been well established that leukocyte populations have the ability to generate pro-inflammatory cytokines (89), neurotoxin or reactive oxygen intermediates (90). Proinflammatory cytokines produced by lymphocytes might be important for cellular, and in particular, for neuronal destruction (89, 91). Cytokines not only play a central role in modulating immune responses and inflammatory reactions, but also can have direct cytotoxic effects.

5.3. Modulating and direct effects of locally synthesized molecules

In several studies, the presence of several cytokines were demonstrated in the brain of BDV-infected rats (37, 92-95). Analysis of brain tissue of BDV-infected rats revealed the presence of various cytokine mRNA's including IL-6, TNF-alpha, IL-1, IFN-gamma, increased levels of IL-2 (37) and, in addition, IL-4 and IL-10 (58; and our own observation). These cytokine mRNA's reached maximum levels at the peak of inflammatory reactions, and decreased dramatically in the chronic phase of the disease. The pattern of IL-4 mRNA indicates a switch from a T-helper1(Th1) cellular immune response to a T-helper2 (Th2) humoral immune response (96). Furthermore, TNF-alpha and -beta as well as IFN expression could be localized in inflammatory cells and predominantly microglial cells.

The observation that the levels of cytokine mRNA's correlated with the degree of inflammatory reactions and severity of neurological signs suggested that the production of certain proinflammatory cytokines may contribute to neurological disease (37). The mechanisms by which cytokines are involved in the disease process are not yet clear, but T cells might recruit activated inflammatory cells through the action of cytokines such as TNF-alpha and -beta and IFN-gamma (97-100). In addition, these cytokines can prime macrophages to produce the inducible reactive oxygen intermediates and reactive nitrogen intermediates (101), that may play an important role in the process of cell destruction (90). Apart from detecting cytokines, chemokines, and the expression of other genes implicated in CNS disease, inflammation and especially in leukocyte recruitment to the CNS, have been analyzed. The mRNA levels of IP-10 and other chemokines were found to immediately precede the inflammatory process. Astrocytes were identified as the major source of IP-10 mRNA (94). Furthermore, COX-2 and CGRP gene expression was drastically up-regulated in cortical neurons of BDV-infected rats, and appeared to have resulted from the inflammatory response in the brain, especially in the presence of macrophage/microglia accumulation (102).

In addition to cytokines, mRNA for inducible nitric oxide synthase (iNOS), which cannot be found in normal brain tissue, is upregulated in the brains of rats infected with BDV (103). The levels of iNOS mRNA correlated not only with the degree of neurological involvement and CNS inflammation, but also with the levels of TNF-alpha , IL-1 and IL-6 mRNA (91), and potential mediators of iNOS expression (90). These observations support the hypothesis that certain cytokines, such as TNF-alpha and IL-1 may participate in the inflammatory process by triggering infiltrating macrophages to generate NO (90). However, immunohistochemical investigations by use of Mab's that distinguish between infiltrating macrophages and resident microglia revealed that macrophages, invading from the blood, seemed to play a minor role, whereas intraparenchymal microglia proliferation and activation was very prominent. Additional *in vivo* studies with inhibitors of cytokines will be necessary to reveal the beneficial and detrimental attributes of cytokine expression during BD and other inflammatory diseases of the CNS.

One of the particularly interesting findings in BD is the chronic phase of the disease which is associated with severe alteration in behaviour (24, 59). Whereas the acute stage of the disease is characterized by neurological dysfunctions, animals in the chronic phase of the disease exhibit increasing passiveness and somnolence and signs of debility and dementia.

Histologically, the chronic phase of BD is characterized by a prominent cortical atrophy and hydrocephalus internus ex vacuo (24, 59). This hydrocephalus does not result secondarily from hydrocephalus occlusus (104) nor from hypoxia, since no signs of vascular damage are found in the brains of BDV-infected rats (54). In untreated rats necrobiotic changes of brain cells are found even in the earliest stages of the disease, and neuronal cell loss is a prominent feature of BD (25). The mechanisms by which virus infections of the CNS cause neuronal damage are not fully known. Studies indicate that in many cases the virus does not directly destroy neurons, but may cause indirect damage by triggering cell-mediated immune response and/or altering neuronal metabolic functions. The presence of inflammatory components, notably T cells and macrophages, which are found in lesions of the central nervous system, frequently characterize neurological disease caused by infection with conventional viruses. Immunopathology may be mediated by cytokines, neurotoxins, radicals and cytotoxic T lmphocytes (CTL). Treatment of BDV-infected rats with anti-CD8 monoclonal antibodies not only reduces or inhibits inflammation as described, but also prevents neuronal degeneration and overt loss of brain substance (54, 67). Interestingly enough, and as mentioned before, in those rats treated with antibodies directed against CD8-positive T cells, CD4-positive T cells and macrophages are still detectable in the brain. In addition, treatment of BDV infected rats with anti-CD8 antibodies results in decreased expression of MHC class I but has no effect on MHC class II expression.

5.4. $CD8^+$ cytotoxic T lymphocytes as effector cells in Borna disease

Since co-expression of MHC class I antigen in association with virus-specific proteins renders cells as targets for toxic CD8-positive CTL (reviewed in 105), the observation that BDV-infected rats have CD8-positive

infiltrates and enhanced MHC class I expression in the brain provides a plausible explanation for the immunopathology seen in this system. Recent experiments employing syngeneic and allogeneic BDV-infected target cells and lymphocyte preparations isolated from the brain of BDV-infected rats showed evidence for the activity of virus-specific classical CD8-positive CTL (35). These experiments also showed weak evidence for the presence of MHC class II restricted CTL among lymphocytes isolated directly from the brain of BDV-infected rats, whereas antiviral activity of classical MHC class I-restricted virus-specific T cells has been found for various virus infections in numerous reports (105-109). In contrast to studies using antibodies to CD8-positive cells, *in vivo* treatment with Mab directed against CD4-positive T cells did not prevent significant loss of brain tissue (35). Virus-specific CD4-positive CTL's might participate in the pathogenic process of late morphological changes in the brain, by direct action on MHC class II-bearing astrocytes which indirectly might result in secondary effects on the neurons (35). However, no considerable MHC class II-restricted cytotoxicity can be demonstrated in the lymphocyte preparations isolated directly from the brain, and the localization of lymphocytes in the brain argues against this possibility (35, 54). Whereas CD4-positive T cells are mainly accumulated perivascularly, CD8-positive T cells are found at significantly higher numbers than CD4+ T cells and macrophages in the brain parenchyma (54). In this localization, the CD8-positive T cells can be found in the direct vicinity of the degenerating neurons. Adoptive transfer experiments with lymphocyte preparations that were isolated from the brain, which did not exert MHC class II-restricted, but high MHC class I-restricted, cytotoxic activity revealed an early onset of severe neurological symptoms, a massive disseminated infiltration to the brain parenchyma with CD8-positive T cells and severe neurodegeneration (110). The involvement of natural killer cells in the local inflammatory reaction in the brain is still unclear; although present in the brain (58, 63). No NK cell activity has ever been demonstrated in brain lymphocyte preparations (35); however, natural killer cells might participate in immunopathogenesis by producing cytokines in the peripheral blood after BDV infection. Therefore, it appears that, in addition to the importance of CD8-positive T cells in triggering a DTH reaction in the brain, CD8-positive T cells are also involved in brain tissue destruction after BDV infection, resulting in organ atrophy, and chronic debility and dementia clinically (35, 54, 67).

Most recently, the role of CD8-positive T cells has been further substantiated by determining the viral target molecule. Employing recombinant vaccinia virus expressing the viral matrix protein p16/gp18, the phosphoprotein p24, the nucleoprotein p40, or the major glycoprotein gp94, the immunodominant viral protein for the CD8-positive T cell-mediated cytotoxic response was determined. In summary, the nucleoprotein p40 represents the major epitope for cytotoxic T cells and there is weak indication for a subdominant epitope on the major glycoprotein gp94 (111). This finding was also verified in the mouse model (112). Finally, the relevant naturally processed target peptide within the nucleoprotein p40 was identified; the peptide ASYAQMTTY at the same time represents the first naturally processed viral target structure for rat CD8-positive T cells (113). Despite the vigorous immune response in the brain, including the activity of the CTL's, BDV also persists in the brain. This finding, however, is not unique since a similar situation has been reported for lymphocytic choriomeningitis virus infection in mice, in which virus clearance is not accomplished despite the presence of CTL activity (114).

An example in which virus was only found transiently in BDV-infected rats has been reported (115). Immunization with a CD4-positive T cell line and subsequent challenge with infectious virus protected the animals from disease. Unfortunately, the mechanism(s) that may give the antiviral impact, i.e., antiviral antibodies, antibodies or clontypic T cells directed against the T cell receptors, or the possible induction of CD8-positive T cells by cytokines produced by these CD4-positive T cells, has not been addressed. In particular, no immunohistological study of the cell types present in the brain, after T cell immunization, e.g., demonstration of CD8-positive T cells, was performed. In addition, in BDV-infected rats, immunopathology and disease could be prevented after transfer of CD4-positive T cells, where it could be demonstrated that this effect was due to the function of cytotoxic CD8-positive T cells, which resulted in the elimination of the virus from the brain (116). A similar observation, possibly based on the same background was observed, when rats that had been immunized with recombinant vaccinia virus expressing the nucleoprotein p40 were challenged with BDV and were found to harbor reduced viral loads (117). Finally, the use of high dose BDV for infection of rats resulted in virus control and elimination from the brain due to the presence of cytotoxic CD8-positive T with specificity for the peptide ASYAQMTTY derived from the nucleoprotein p40 of BDV (123).

The BD in rats provides a model that supports the notion that actions of CD4 and CD8 subsets are required for antiviral DTH responses. By which mechanisms CD8-positive T cells cause tissue destruction is still unsolved. The CD8-positive T cells are potent producers of cytokines such as IFN-gamma (118, 119) which activates macrophages and induces a DTH reaction, including the activation of nonspecific host inflammatory cells that finally results in tissue injury. To what extent other deleterious cytokines and radicals are involved in the tissue destruction is not yet clear. Alternatively, virus-specific CD8-positive T cells might kill their target cells after activation of their cytolytic mechanims, including the action of perforin and other effector molecules (108, 120-122). In this respect, it is of interest and importance that up-regulation of perforin mRNA in the brains of BDV infected rats has been detected, in parallel with the first signs of tissue destruction and the presence of the CD8-positive T cells (110). In conclusion, BD in rats appears to be a CD4-positive T-cell-dependent immunopathological disease, in which CD8-positive T-cells and/or CD8-positive T cell-mediated cytodestructive mechanisms are operative, leading to tissue destruction, organ atrophy, and clinically to organ dysfunction and disease.

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