DIPLOMARBEIT

Titel der Diplomarbeit
Cell Death in Viral Encephalitis Diseases

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angestrebter akademischer Grad
Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt: A 490
Studienrichtung lt. Studienblatt: Diplomstudium Molekulare Biologie
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Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system resulting from a viral infection. JC virus (John Cunningham) is known to cause the demyelinating lesions in the white matter, but how oligodendrocyte death is really initiated during disease course is still under question. Besides the demyelination pathology, inflammation can be detected in the brain parenchyma of PML-patients. Though it is not established how the immune response including both activation of CD8+ cytotoxic T-lymphocytes and granzyme B release is involved in the pathogenesis. Therefore histochemical and immunohistochemical experiments were performed in order to get a better insight. The results of the investigations showed that the immune response is playing a minor role in PML and that hence induction of oligodendrocyte death is not likely to be initiated by to granzyme B release. In fact, no evidence was found that apoptosis is the deathly mechanism causing oligodendrocyte death. Therefore, other death inducing pathways were checked that would possibly give an explanation how CNS damage is caused. It was found that the key events of parthanatos, a rather recently described mechanism, were also detectable in the JC virus infected oligodendrocytes. These events included accumulation of poly(ADP-ribose) (PAR) in the cytosol and translocation of apoptosis inducing factor (AIF) out of the mitochondria into the nucleus, where it is capable of inducing DNA-fragmentation leading to cell death. In order to compare the results to other viral encephalitis diseases the same experiments performed on the PML-samples were also repeated on herpes simplex virus (HSV) encephalitis samples and cytomegalovirus (CMV) encephalitis samples. Interestingly, differences were found when comparing PML to HSV encephalitis and CMV encephalitis. In the latter ones the immune response seems to be more central and apoptosis was found to be the cell death inducing pathway and leading to CNS damage in those diseases.
2. Zusammenfassung


Mit diesen Ergebnissen konnte eine Erklärung für den Zelltod der Virus-infizierten Oligodendrozyten gefunden werden und es wurde gezeigt, dass unterschiedliche Mechanismen hinter der Entstehung des Schadens im Gehirn in den verschiedenen Virusencephalitiden stehen.
Introduction

3. Introduction

3.1. Acute viral encephalitis

Encephalitis is an inflammatory process affecting the brain parenchyma. This inflammation is further linked to brain dysfunction. Generally, the triggering causes as well as the presentation of encephalitis are widely ranging. So, encephalitis can occur as an acute disease or a chronic disease. Since more information on acute viral encephalitis diseases is given later, chronic encephalitis is just shortly mentioned here. There are various different types described, but to give names, Rasmussen’s encephalitis like subacute sclerosing panencephalitis (a late complication of measles infection), constitute to the group of chronic encephalitis.

Another possible division of the different encephalitis diseases can be made according to the underlying causative factors. One possible group consists of encephalitis that results from an aberrant immune response, leading to development of autoimmune encephalitis. Examples for this kind of disease are acute disseminating encephalomyelitis (ADEM), acute hemorrhagic leukoencephalopathy (AHLE) and brain stem encephalitis. The other subtype can be termed infection-related encephalitis and might be the result of an infection with bacteria, viruses and parasites. The most prominent infectious agents are viruses which are likely to induce formation of acute viral encephalitis (Stone and Hawkins, 2007). The usual presentation of the disease is found to be fever, headache, altered consciousness, focal neurology and in some patients also seizures are occurring. If those symptoms are present an immediate start with treatment is beneficial for the outcome, since it is reducing the extent of brain damage and favoring survival (Steiner et al., 2005; Stone and Hawkins, 2007). Prominent viral encephalitis diseases are herpes simplex virus encephalitis (HSV), varicella-zoster virus encephalitis (VZV), Epstein-Barr virus encephalitis (EBV) as well as cytomegalovirus encephalitis (CMV). Another disease resulting from a viral infection of the central nervous system is progressive multifocal leukoencephalopathy (PML). PML is caused by the JC virus, which is present in 80% of the population, but is only being activated and leading to demyelination in the white matter in immunocompromised patient (Weissert, 2011).

Since samples out of PML, CMV and HSV patients were most interesting and intensively studied in this project, more detailed information on these three diseases is given next.
3.1.1. Progressive multifocal leukoencephalopathy (PML)

Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system (CNS). PML was first described in 1958 in immunosuppressed patients suffering from chronic lymphatic leukemia and Hodgkin’s disease (Astrom et al., 1958). In the early 1970s culturing of the virus isolated from a PML patient’s brain was succeeded and virus characterization was possible. It was found that a polyomavirus, called “JC” virus, named after the patient (John Cunningham) it was isolated from, was the underlying cause of the deadly disease (Padgett et al., 1971). Polyomaviruses are a family of double stranded DNA viruses. They have an icosahedral capsid which consists of three viral proteins, VP1, VP2 and VP3. Of these, VP1 is the most prominent protein to be recognized as viral particle by the immune system (Bag et al., 2010; Goldmann et al., 1999; Goldmann et al., 2000). PML is an opportunistic infectious disease (Berger and Houff, 2009; Weissert, 2011) and it mainly emerges in patients suffering from immunosuppression such as patients with lymphoid malignancies, organ transplant recipients and patients with AIDS (Wuthrich et al., 2006). The latter accounts for more than 80% of all PML cases (Koralnik et al., 2004) and up to 5% of AIDS patients were found to develop PML at the beginning of the AIDS epidemic (Berger et al., 1987). In 2005, PML was also seen to develop in multiple sclerosis and Crohn’s disease patients that were receiving immunomodulatory therapy, namely natalizumab, an mAb against CD49a (Kleinschmidt-Demasters and Tyler, 2005; Langer-Gould et al., 2005). In addition, in 2008, PML was seen to develop after treatment with rituximab, a mAb against CD20 (Carson et al., 2009). Interestingly in many countries up to 92% of the adults are seropositive for JC virus (Walker and Padgett, 1983). Although the virus is widely spread in the population, the incidence of PML is rather low.

During development of PML three phases can be distinguished. The first phase is the primary infection with JC virus. This process is usually subclinical and asymptomatic and happens mostly during childhood (Bag et al., 2010; White and Khalili, 2011). In the following phase, the virus is thought to be latently present in the body. At which sites the virus usually resides is not totally clear, but it was detected in the kidney (Yogo et al., 1990), tonsils (Kato et al., 2004; Monaco et al., 1998), peripheral blood cells (Dorries et al., 1994), the bone marrow (Tan et al., 2009a) and also in the brain of individuals who weren’t suffering from PML (Mori et al., 1991). During the third and last phase, which is preferentially initiated in immunosuppressed individuals, the virus gets reactivated and appears within the CNS. How the dissemination to the brain is achieved is not really known, but hematogenous spreading is a possible mechanism (Bag et al., 2010; Weber, 2008). Also the timing when the virus enters the brain is not clear. It might be that the virus settles in the brain during primary infection, but it is also possible that JC virus spreads to the brain only during the reactivation process.

Interestingly, two different types of JC virus have been identified until today and the differences of these virus types are seen in a key regulatory region, a noncoding control region (NCCR). This part of the genome seems to be of great importance as it regulates the viral replication as well as early and late
transcription events (White and Khalili, 2011). One of these virus types, the so called “archetypal” form, typified by the CY stain of JC virus, is predominantly present in the kidney, urine and sewage (Yogo et al., 1990), whereas the other form, known as the “prototypical form”, also “PML-type” of JC virus is found in PML and is also identified by a specific stain, called the Mad-1 stain (Frisque et al., 1984).

Knowing this, different options for development of PML are possible. One possibility is a genetic switch between the different virus types resulting in a kind of neuroadaptation (White and Khalili, 2011) combined with hemorrhagic spreading to the brain. The other possibility would be a superinfection of both viruses, whereas only the “PML-type” JC virus manages to enter the brain. It can be easily seen that further investigations need to be done until all the questions on how JC virus is acting in the body and manages to infect the CNS are resolved.

Figure 1 The typical features seen in patient's brains suffering from PML: a scan of paraffin-embedded section stained with luxol fast blue (Klüver-Barrera); b enlarged picture of a typical demyelinating white matter lesion resulting from oligodendrocyte death stained with luxol fast blue (40x magnification); c white matter lesion stained with an antibody targeting JC virus and it can be seen that the virus infected cells are located at the edge of the lesion (40x magnification); d JC virus infected oligodendrocyte showing a typical swollen shape (250x magnification); e JC virus infected oligodendrocyte undergoing cell death (250x magnification); f+g bizarre shaped JC virus containing astrocytes (250x magnification)
The symptoms resulting from PML-development are mainly neurological. Patients show neuropsychological deficits, apraxia and dementia and suffer from visual deficits as well as motor problems (Berger and Major, 1999; Krupp et al., 1985; Weissert, 2011). As already indicated by the name, the disease course is progressive and therefore, unless treated, the patients remain in an immunocompromised state and die within months (Weissert, 2011). Pathological hallmarks of PML include virus containing oligodendrocytes which are located around demyelinating white matter lesions, bizarre shaped virus infected astrocytes in the centre of burnt out lesions and an inflammatory response consisting of lymphocytes and phagocytic macrophages (Richardson, Jr. and Webster, 1983) (see Figure 1).

To avoid a fast death, an immediate reaction is necessary, but unfortunately there is no therapeutic agent available that is specifically harming the JC virus (Marshall and Major, 2010). Various trials are performed with drugs like nucleoside analogues, antipsychotics and cytokines. HIV-positive patients are treated with cART, an antiviral therapy, which is preventing HIV from replication and due to a higher immune response the outcome for PML is better as well. So far, only reconstitution of the host immune system seems to delay the deathly outcome of PML (Weissert, 2011).

Another disease that is sharing some features with PML and is also induced by the infection with JC virus is PML immune reconstitution inflammatory syndrome (IRIS). A difference between PML and PML-IRIS is found in the disease development, since PML develops during immunosuppression, whereas PML-IRIS develops in cART treated HIV-patients during recovery of the immune system (Weissert, 2011). Fortunately, unlike in PML, treatment with steroids is very effective in PML-IRIS (Tan et al., 2009b). Though PML and PML-IRIS are the most common forms of disease, there are other forms in which JC virus is identified to be the underlying cause. These diseases are referred to as JCV encephalopathy (Sandyk, 1983), JCV meningitis (Blake et al., 1992) and JCV granular cell neuronpathy (Koralnik et al., 2005) (Bag et al., 2010).

3.1.2. Cytomegalovirus encephalitis (CMV)

Cytomegalovirus (CMV) is a member of the human herpesviridae family, whose members are known to be double stranded DNA viruses. Together with human herpesvirus-6 and -7, CMV is assigned to the subgroup of beta herpesvirinae. First contact with CMV is usually in the first 2 decades of life (Zamora, 2011), whereas primary infection can also very often happen intrauterine, congenitally, perinatally or postnatally (James et al., 2009). It has been tried to erase CMV from the immune system through cell-mediated immunity or establishment of neutralizing antibodies. However, the virus seems to escape and remains latently in the body. CMV can reside in many different cells like hematopoietic progenitor cells, leukocytes, macrophages, peripheral blood cells, as well as endothelial cells and fibroblasts (Zamora, 2011). As already mentioned, CMV can infect endothelial cells, which are also located in the brain and the choroid plexus. Consequently, CMV can infect astrocytes near the
epithelium and can further use cerebrospinal fluid (CSF) to be transferred to the ependymal surfaces (James et al., 2009). Reactivation of JC virus is facilitated in immunocompromised individuals and it seems that the situation for CMV is the same, since it is also found to be mainly infectious in HIV positive patients (Quinnan, Jr. et al., 1984) or patients undergoing organ transplantations (Booss and Kim, 1989). Upon reactivation, CMV among others, can induce different diseases like CMV retinitis, colitis, pneumonitis and encephalitis (Rafailidis et al., 2008). In this thesis CMV encephalitis was particularly studied. Histopathologically, CMV encephalitis often shows inflammation around the ventricles. Due to spreading of the virus with CSF, in the parenchyma focal necrotic lesions as well as microglia nodules can be seen. Infected cells (either neurons or astrocytes) typically are enlarged and contain virus inclusions (see Figure 2). Due to the typical morphology, those cells are commonly known as “owl eyes” (Arribas et al., 1996) (Bestetti et al., 2001).

Figure 2 Labeling of CMV infected cells with immunohistochemistry; A the typical “owl’s eyes” resulting from CMV infection can be easily distinguished from the non-infected cells, still there are also cells present that contain CMV and are not shaped in the typical “owl eye” fashion (250x magnification); a in the small insert picture an “owl’s eye” containing viral inclusions is shown (400x magnification)
Patients with CMV spreading to the CNS usually present with fever, altered mental status, progressive confusion and sometimes impairment of memory and dementia. Rather rarely seen are patients suffering from seizures (Post et al., 1986).

Most patients receive gancyclovir or foscarnet as antiviral therapy. Gancyclovir is a nucleoside analogue, whereas foscarnet is a pyrophosphate analogue (Arribas et al., 1996) In some cases, patients are treated with a combination of both substances (Silva et al., 2010).

3.1.3. Herpes simplex virus encephalitis (HSV)

Herpes simplex virus (HSV) is a double stranded DNA virus and constitutes to the family of human herpesviridae. There are two different types of HSV infectious for human, HSV type-1 and HSV type-2. Both types are known as alaphaherpesvirinae (Zamora, 2011). Generally, both HSV type-1 as well as HSV type-2 are neurotropic and are capable of inducing HSV encephalitis. Still incidence rates show that HSV type-1 is much more common than HSV type-2, which only accounts for 10% of HSV encephalitis (Stone and Hawkins, 2007). A difference between the two virus types is that HSV type-1 is orally transmitted whereas HSV type-2 gets spread through genital transmission (Corey and Spear, 1986). Primary infection is often subclinical and there are no symptoms detected. However, if there are effects seen, distinction between HSV type-1 and type-2 infection is possible. Manifestations of HSV type 1 infection are usually seen as fever blisters on the labia, whereas insults from HSV type 2 infections are mainly presented on genital and perianal areas as painful vesicles and ulcers (Conrady et al., 2010) (Zamora, 2011). Though being a rather rare complication HSV can spread to the CNS and cause encephalitis. Since HSV is a neurotropic virus it preferentially infects neurons. For HSV type-1 there is data available that confirms that this virus is entering the cell via binding to heparan sulfate proteoglycan and that the virus is residing in the neurons, which are used as hosts (Conrady et al., 2010; Shieh and Spear, 1994; WuDunn and Spear, 1989). Accordingly the virus can spread via retrograde axonal transport and infect the nucleus of sensory ganglia (Shimeld et al., 2001). Like JC virus also HSV changes to a latent state after the primary infection and resides in the host’s sensory ganglia cells until it gets fully reactivated again (Bertke et al., 2009). The exact circumstances for viral reactivation are not totally understood, but it seems that stress to the host immune system is a beneficial factor for the virus’s reactivation.

There can be found a lot of information on how the host immune system is acting in order to destroy HSV, but to completely understand the effects resulting from the immune response further investigations will be needed. Until today it became clear that HSV type-1 is triggering activation of the innate immune system through toll-like receptors (TLRs), which lead to type I interferon production, initiation of an inflammatory response and an according activation of the adaptive immune system. Nevertheless, remains the question, what factors lead to this extent of CNS pathology in HSV encephalitis. It is thought that due to extensive activation of the immune system the virus is eliminated.
in the peripheral nervous system, but escapes to the CNS. Possible explanations are activation and release of toxic substances out of microglia and astrocytes which lead to CNS insult or the recruitment of activated leukocytes into the brain, which then release cytotoxic molecules and therefore induce cell death of infected cells (Conrady et al., 2010).

Results from the CNS damage can be seen as symptoms in the patients. Usually, HSV encephalitis is presented with headache, fever and an altered consciousness, whereas the resulting long-term consequences can be memory impairment, abnormalities in behavior and personality, epilepsy, anosmia (lack of perceiving odors) and dysphasia (McGrath et al., 1997; Stone and Hawkins, 2007). Interestingly there are differences in the presentation seen in adults compared to neonates and young children. In latter ones, HSV encephalitis is found to be more diffuse and in multiple locations (Batnitzky et al., 1986).

Fortunately, HSV encephalitis can be treated very successfully with acyclovir (Skoldenberg et al., 1984; Whitley et al., 1986). Only if patients are infected with an acyclovir-resistant strand, they are treated with different drugs like cidofovir or foscarnet (Losada et al., 2002; Superti et al., 2008).

Histological features, that are mostly found in adult HSV encephalitis brains are necrotic and hemorrhagic properties mainly located in the frontal or temporal lobe (Conrady et al., 2010; Kleinschmidt-Demasters et al., 2001). In ABC-studies it is shown that the main infected cells with HSV are neurons, astrocytes and oligodendrocytes, whereas the antigen can be found in the nucleus and the cytoplasm of those cells (Benjamin and Ray, 1975) (Kumanishi and Hirano, 1978).

### 3.2. Cell death

Cell death is a very important event in cells and just during the last years it has become clear that there are many different mechanisms with different morphological features involving various biochemical pathways. A prominent way of cell death is apoptosis, which is willingly induced by the cell by activating a special set of proteolytic enzymes (see below). Other possible mechanisms are necrosis, autophagic cell death, mitotic catastrophe and parthanatos, just to name a few. Since there is no common classification of the different pathways, mixing the different terms is an easy occurring mistake. Therefore a recommendation on the nomenclature of cell death is available, which aims to find a consensus on all the different mechanisms and terms used (Galluzzi et al., 2011).

Since only necrosis, apoptosis and parthanatos are of greater importance for this topic, only these three mechanisms are further described.
3.2.1. Necrosis

Necrosis is a form of programmed cell death, but it is usually not willingly induced by the cell, since its induction usually depends on extrinsic factors. This mechanism can be initiated under conditions like lack of blood supply, trauma or as a response to acute insult. Unlike in apoptosis, during necrosis cell content is released, triggering an inflammatory immune response, which results in direct damage to the surrounding tissue (Alberts et al., 2007).

Typical morphological features occurring during necrosis are cell swelling and rupture of the cell membrane, whereas different from apoptosis, compaction of chromatin is not seen (Andrabi et al., 2008) (see Figure 3).

Figure 3 hematoxylin stainings; a shows healthy cerebellum-structure in which the cells are well-shaped and not showing any signs of damage (100x magnification); b shows necrotic tissue in which the cells are damaged and destroyed (100x magnification)

3.2.2. Apoptosis

Apoptosis is probably the most prominent and the best studied mechanism of programmed cell death occurring in an organism. Apoptosis plays a central role during development, but also in adult organisms, in which the process is induced in a strict regulated manner, keeping cell division and cell death in exact balance. Characteristic features that can be detected during apoptosis are cell shrinking, cell condensation, collapse of the cytoskeleton, a disassembling of the nuclear envelope, as well as chromatin condensation and DNA fragmentation (Alberts et al. 2007).

Galluzzi et al. described that until now four different modes of apoptosis can be identified. These are: 1) caspase-dependent intrinsic apoptosis, 2) caspase-independent intrinsic apoptosis, 3) extrinsic apoptosis induced by death receptors and 4) extrinsic apoptosis induced by dependence receptors. As
the names already indicate in these modes a distinction is made between the inducing factors (intrinsically or extrinsically) and a distinction in the involvement of caspases (Galluzzi et al., 2011). Generally, caspases are known as aspartate-specific cysteine proteases and as members of the interleukin-1β-converting enzyme family. Up to now, 14 different caspases have been identified and they all share common features, like being synthesized as procaspases orzymogens. After activation through proteolysis, which can result from autoactivation or due to cleavage by another caspase, they build up the active heterotetrameric structure (Fan et al., 2005) (Launay et al., 2005). Although caspases show homologies, they exhibit different functions and accordingly a division into three subtypes can be done. The first group of caspases consists of enzymes that are known as apoptosis activators. They are mainly active at the start of the apoptotic signaling cascade and (because they act as activators) of downstream caspases. The second group consists of caspases that act as executioners or effector caspases and are activated in a rather late phase of the apoptotic signaling pathway. The last group consists of caspases that are involved in inflammatory processes which are not directly linked to apoptotic cell death (Fan et al., 2005).

Since in the context of PML extrinsically induced apoptosis by cytotoxic T-lymphocytes (CTL) and granzyme B is the mechanism that is studied, a short overview on this pathway is given. Cytotoxic T-cells are capable of inducing apoptosis in two different ways. Either CTL activate the apoptotic signaling cascade due to the interaction of TNF superfamily ligands or Fas/CD95 with their receptors or they release granzymes, which attack the cell membrane of the target cell and consequently induce activation of the caspase cascade due to cleavage of specific proteins (Afonina et al., 2010; Cullen and Martin, 2008; Russell and Ley, 2002). Granzyme B is known to act in two ways (shown in Figure 4). Since granzyme B is a CTL/NK protease, it can cleave substrates and was found to preferentially cleave after aspartates. Due to this function granzyme B is able to cleave caspases, especially caspase-3, and is therefore activating the caspase signaling cascade (Adrain et al., 2005; Afonina et al., 2010). The other possibility is, that granzyme B is initiating the mitochondrial/apoptosome pathway by cleaving Bid, which induces oligomerization of Bax and/or Bak, which consequently leads to pore formation in the mitochondrial membrane. Through those holes proteins, such as cytochrome c, can leave these organelles and can induce the formation of the apoptosome, which also results in caspase-activation and cell death (Afonina et al., 2010).
Figure 4 Apoptotic pathways induced by granzyme B; two mechanisms are shown; first one is the direct cleavage of caspase-3 by granzyme B which leads to activation of the signaling cascade. In the second way granzyme B cleaves Bid, which forms truncated Bid (tBid), which accordingly leads to Bax/Bak pore formation in the mitochondrial membrane. Through those pores cytochrome c can be released and induces the formation of the apoptosome consisting of Apaf-1 and caspase-9 which then induces further activation of the caspase signaling cascade (Afonina et al., 2010)

It is not clear, whether extrinsically induced apoptosis by CTL due to granzyme B release is really inducing oligodendrocyte death in PML patients. Currently, there are two studies available that are describing the presence of lymphocytes in the CNS (Aksamit et al., 1990; Wuthrich et al., 2006). Wüthrich et al. performed a study in which autopsy tissue out of HIV+ and HIV-patients was investigated with special interest of lymphocyte location towards JC virus infected oligodendrocytes. It was found that there are positive correlations of CD8⁺ cytotoxic T-cell, which were shown to be the most prominent subtype of immune cells located in the CNS parenchyma, with the JC virus infected cells. Therefore they argue that this apposition indicates a possible role of CTLs in killing oligodendrocytes, however it is also mentioned in the paper that due to usage of autopsy tissue there are experimental limitations resulting. Accordingly, it was not possible to distinguish if those CTL
found in the CNS parenchyma and closely located to the JC virus positive cells are really recognizing JC virus specific proteins or if their recruitment results from other attractants (Wuthrich et al., 2006). A major drawback of this paper is that they did not show that CD8\(^+\) T cells in the neighborhood of infected cells actually contain cytotoxic granules (no granzyme B or perforin stainings), nor did they reveal any indication that these cytotoxic T cells are polarized and actually release cytotoxic granules. Therefore further studies are needed to really identify the specific involvement of cytotoxic T-lymphocytes in PML.

### 3.2.3. A new cell death mechanism; Parthanatos

Recently, it was found that there exists another deathly pathway besides the well known cell death mechanisms such as apoptosis, necrosis and autophagy. This pathway was termed parthanatos. Parthanatos definitely differs from the cell death mechanisms listed above and induces cell death in a caspase-independent manner (Andrabi et al., 2008). The name of the mechanism already includes one of the two most central molecules constituting to the executive function of this pathway. The molecule, which is referred to here, is poly (ADP-ribose) or shortly PAR. This molecule is formed as a side product during the poly-adenylation reaction catalyzed by the enzyme poly (ADP-ribose)-polymerase-1 (PARP-1) (Smith, 2001). PARP-1 is usually activated as a response to DNA damage. If the enzyme is activated, NAD\(^+\) is used as substrate and ADP-ribose polymers are attached to either acceptor proteins (heteromodification) or are transferred on PARP-1 (automodification) (D’Amours et al., 1999; Virag and Szabo, 2002). The extent of the damage occurring on the DNA has an influence on how the enzyme functions, since under rather low stress conditions the damage is repaired, whereas under high stress conditions with extensive DNA-damage like in stroke or traumatic brain injury cell death is induced (Virag and Szabo, 2002). Due to the overactivation of PARP-1, high amounts of PAR are formed and the molecule accumulates in the nucleus (Andrabi et al., 2006). Somehow, PAR manages to translocate to the cytosol, but how this is achieved is presently unknown. At this point, PAR is highly enriched in the cytosol and this is thought to be the starting point of the cell death cascade involved in parthanatos (shown in Figure 5). Interestingly, PAR colocalizes with mitochondria and these organelles are already known to contain cell death effector molecules, such as cytochrome c and the apoptosis inducing factor (AIF) (Beal, 2005). AIF, when first identified, was thought to be involved in cell death (Susin et al., 1999), but later was found to be also important in energy-generating processes (Pospisilik et al., 2007). In the context of parthanatos, AIF plays a central role (Yu et al., 2006). The point of AIF release is generally handled as the commitment point for cell death in parthanatos (Andrabi et al., 2006). The exact mechanism, how AIF is released from the mitochondria, is still not totally solved, but it was found that AIF was released in an uncleaved form, as a 62kDa protein (Wang et al., 2009). In the brain, AIF seems to be associated with the outer
mitochondrial membrane and not to be only present in the intermembrane space, where it is located to the inner membrane. It was suggested that the localization at the outer membrane would enable AIF to be released very quickly during parthanatos and the amount of the molecule at this site would be sufficient to induce cell death (Yu et al., 2009). Recently, it was described that PAR is binding to AIF which is located at the outer mitochondrial membrane and due to this interaction AIF is released (Wang et al., 2011). Once free, AIF is present in the cytosol and is translocated to the nucleus where it is capable of inducing large-scale DNA fragmentation, chromation condensation and cell death (Krantic et al., 2007; Modjtahedi et al., 2006). Generally, it is said that this cell death mechanism is independent of caspases, however in the late phase of parthanatos caspases get activated, as well. But since AIF is the deadly inducing factor in this pathway and caspases are only being activated at the late phase, they are not exhibiting an executioner function (Yu et al., 2002; Yu et al., 2006).

Figure 5 Signaling involved during induction of poly(ADP-ribose) polymerase-1 (PARP-1) dependent cell death (Parthanatos): Due to DNA damage, overactivation of PARP-1 an extensive PAR formation is induced. PAR usually present in the nucleus, is translocated by an unknown mechanism to the cytosol, where if AIF-binding is possible, AIF release out of the mitochondria and translocation to the nucleus is achieved. AIF in the nucleus leads to DNA fragmentation and cell death (Wang et al., 2011)

Parthanatos or parthanatos-related events have already been described for some diseases. For example, it has been shown, that an increased amount of poly-ADP-ribosylated nuclear proteins is present in Alzheimer’s disease (Love et al., 1999). In addition, there is data available that poly-ADP-ribose polymerase-1 plays a crucial role in Parkinson’s disease (Mandir et al., 1999; Outeiro et al., 2007).
The paper of Veto et al which suggests that demyelination resulting from oligodendrocyte death in pattern III lesions of multiple sclerosis might be induced by parthanatos, is interesting regarding this topic. In this article experiments were performed using a mouse model, in which primary demyelination was induced by treatment with cuprizone. Mice treated with PARP-1 inhibitors revealed decreased demyelination. The conclusion was made that a caspase-independent cell death involving translocation of AIF to the nucleus was coming into account. Furthermore, it was concluded that inhibition of PARP-1 reduced death of oligodendrocytes, since PAR accumulation is avoided and therefore parthanatos would not be initiated (Veto et al., 2010).

3.2.4. Current view on cell death in progressive multifocal leukoencephalopathy

Despite that it is a central pathological feature resulting in massive damage in patients, until now there is little data available on how oligodendrocytes actually die in PML. There are two studies published which are dealing with the question of oligodendrocyte death. Interestingly, these two papers are claiming different, even controversial mechanisms leading to cell death. One paper favors apoptosis as the death inducing mechanism (Richardson-Burns et al., 2002) whereas in the other study it is argued that apoptosis is not the inducer of oligodendrocyte death since anti-apoptotic proteins are found in the infected oligodendrocytes (Pina-Oviedo et al., 2007). Since the main topic of this thesis is to deal with the mechanism of oligodendrocyte death, the present view in this field will be shortly reviewed.

The paper published by Richardson-Burns S.M. et al. in 2002 shows that oligodendrocytes are dying due to apoptosis. The study was performed on 8 specimen gained out of PML-patients, whereas 6 of them were HIV-positive and 2 were non-HIV patients. The methods used were immunohistochemical stainings with markers for the JC virus and for activated caspase-3, TUNEL staining and in situ hybridization. The results of the study show that virus infected oligodendrocytes are dying due to apoptosis, since cells are found showing a double labeling for TUNEL-staining and the virus marker, an anti-SV40 large T-antigen antibody. Interestingly, it was not possible to see the typical morphological changes, like chromatin condensation, usually appearing in a cell during apoptosis in the JC virus infected oligodendrocytes, though they are said to undergo this form of cell death in this paper. The authors also claim to find virus infected oligodendrocytes being positive for activated caspase-3, though they were not able to perform a double staining for activated caspase-3 together with JC virus. Further it is argued that the number of virus infected oligodendrocytes and the apoptotic cells found in the area of interest are approximately the same and that therefore the conclusion can be made that all apoptotic cells were JC virus positive since virus infection is inducing the mechanism of apoptosis (Richardson-Burns et al., 2002).

On the other hand, Pina-Oviedo et al. are describing an anti-apoptotic mechanism for JC infected oligodendrocytes. They point out, that the JC virus is capable of prohibiting apoptosis, the
Introduction

normally induced host defense mechanism against virus infected cells, by the newly induced expression of the anti-apoptotic protein survivin. This protein is known to be highly expressed during embryonic development, but in adults and fully differentiated cells, it cannot be detected anymore and is consequently thought to be silenced in those cells (Li and Altieri, 1999). Pina-Oviedo et al. used 20 autopsy samples of PML patients and performed immunohistochemical stainings for the presence of survivin. Interestingly they found that the anti-apoptotic protein is present in the JC virus infected cells and also confirm this by performing a double labeling for the virus and survivin. In oligodendrocytes, survivin is detected in the nucleus and in the bizarre shaped astrocytes the protein is mainly located to the cytoplasm. To further proof the effects of survivin cell culture studies were performed. In those studies results are gathered that expression of survivin differed greatly in JC virus infected cells compared to non-JC virus infected cells. These observations are also seen in the autopsy tissue. To proof that survivin is really prohibiting apoptosis, there were comparative studies performed, in which apoptosis has been induced in JC virus infected cells, containing survivin, and control cells, in which survivin is absent. The results indicated that apoptosis is induced to a much higher extent in control cells compared to JC virus positive cells, which are rather unlikely to die. Due to all of these findings the authors claim that the virus inducing PML is capable of reinducing the expression of survivin. They argue as well that this mechanism would be beneficial for the life cycle of JC virus, because with survivin having anti-apoptotic effects, oligodendrocyte death is being prohibited and therefore the virus is able to finish its life cycle and can consequently replicate in higher amounts (Pina-Oviedo et al., 2007). By showing this data the authors found a nice concept how the virus might act, but still the central question of how oligodendrocytes are really dying is not clearly resolved.

Having all the interesting and still controversial information given in those two papers cited above, it is rather difficult to distinguish which mechanism is really coming into account and inducing this extent of damage in the patient’s brains suffering from PML. Therefore a critical approach seems to be central for further investigations which definitely need to be done.
4. Materials and Methods:

4.1 Study-samples

In this study formalin-fixed and paraffin-embedded human autopsy tissue of patients suffering from progressive multifocal leukoencephalopathy (PML) (n = 8) collected by Professor Jellinger, patients suffering from hepatitis simplex encephalitis (HSV) (n = 5) and of patients suffering cytomegalovirus encephalitis (CMV) (n = 7), were the basic study samples of this work.

All of the PML-patients suffered from HIV-infection as a primary disease. The disease duration for PML varied widely in all of the cases and the regions where the samples were taken from were also widespread. Unfortunately there was no information available about the patient’s history in the HSV-cases. For the CMV-cases some data, but not the complete patient’s history was available. For more detailed information see Table 1.

<table>
<thead>
<tr>
<th>Sample-number</th>
<th>Disease</th>
<th>Age</th>
<th>Male/Female</th>
<th>Disease Duration (wk)</th>
<th>Sample Area</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-93-2</td>
<td>PML (HIV)</td>
<td>45</td>
<td>Male</td>
<td>118</td>
<td>Lenticular nucleus</td>
<td>Pulmonary edema</td>
</tr>
<tr>
<td>47-93-3</td>
<td>PML (HIV)</td>
<td>42</td>
<td>Male</td>
<td>20</td>
<td>Pons</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>97-93-8</td>
<td>PML (HIV)</td>
<td>50</td>
<td>Male</td>
<td>20</td>
<td>Parietal lobe right</td>
<td>Pulmonary edema</td>
</tr>
<tr>
<td>103-93-8</td>
<td>PML (HIV)</td>
<td>39</td>
<td>Male</td>
<td>15</td>
<td>Parietal lobe left</td>
<td>Acute respiratory insufficiency</td>
</tr>
<tr>
<td>278-93-6</td>
<td>PML (HIV)</td>
<td>29</td>
<td>Male</td>
<td>19</td>
<td>Parietal lobe left</td>
<td>Cachexia</td>
</tr>
<tr>
<td>291-93-5</td>
<td>PML (HIV)</td>
<td>49</td>
<td>Female</td>
<td>5</td>
<td>Parietal lobe right</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>358-93-6</td>
<td>PML (HIV)</td>
<td>39</td>
<td>Male</td>
<td>27</td>
<td>Parietal lobe left</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>359-93-7</td>
<td>PML (HIV)</td>
<td>34</td>
<td>Male</td>
<td>&lt;18</td>
<td>Pons</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>330-82-4</td>
<td>HSV</td>
<td>9</td>
<td>Female</td>
<td>1.5</td>
<td>-</td>
<td>Acute respiratory insufficiency</td>
</tr>
</tbody>
</table>
Table 1 Samples of PML-, HSV- and SSPE-patients included in the study

<table>
<thead>
<tr>
<th>Sample</th>
<th>Virus Type</th>
<th>Age</th>
<th>Gender</th>
<th>Duration</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>67-83-2</td>
<td>HSV</td>
<td>40</td>
<td>Male</td>
<td>3.5</td>
<td>Necrotic hemorrhagic pneumonia</td>
</tr>
<tr>
<td>204-83-4</td>
<td>HSV</td>
<td>61</td>
<td>Female</td>
<td>1.5</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>529-84-6</td>
<td>HSV</td>
<td>37</td>
<td>Male</td>
<td>2.3</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>237-91-5</td>
<td>HSV</td>
<td>76</td>
<td>Male</td>
<td>3.15</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>364-92-4</td>
<td>CMV (Toxoplasmosis)</td>
<td>40</td>
<td>Male</td>
<td></td>
<td>acute cardiovascular failure</td>
</tr>
<tr>
<td>455-92-7</td>
<td>CMV</td>
<td>48</td>
<td>Male</td>
<td>&lt;5</td>
<td>Pneumonia, Sepsis</td>
</tr>
<tr>
<td>559-92-11</td>
<td>CMV (HIV, Toxoplasmosis)</td>
<td>33</td>
<td>Male</td>
<td>20</td>
<td>Cerebellum right</td>
</tr>
<tr>
<td>234-93-4</td>
<td>CMV (B-cell lymphoma)</td>
<td>28</td>
<td>Male</td>
<td>11</td>
<td>Pneumonia, Sepsis</td>
</tr>
<tr>
<td>430-93-6</td>
<td>CMV</td>
<td>35</td>
<td>Male</td>
<td>15</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>62-94-9</td>
<td>CMV (B-cell lymphoma)</td>
<td>32</td>
<td>Male</td>
<td>&gt;4</td>
<td>Medulla oblongata</td>
</tr>
<tr>
<td>118-94-5</td>
<td>CMV (HIV)</td>
<td>55</td>
<td>Male</td>
<td>9</td>
<td>Hippocampus right</td>
</tr>
</tbody>
</table>

4.2 General staining procedures

4.2.1 Deparaffination

Since paraffin-embedded sections were used as a basis for the study, deparaffination needed to be performed for all of the different labeling methods used and described below.

Deparaffination was done by incubating the slides 2 times in xylene for 15 minutes and accordingly rinsing them twice in 96% ethanol. Depending on the staining blocking of endogenous peroxidase activity was performed by leaving the sections in 0.02% H2O2-methanol for 30 minutes. This blocking step was only needed in immunohistochemical stainings using peroxidase activity as a detection method. In all other labeling methods, (especially for confocal microscopy), this incubation time was
skipped. Next rehydration followed and was achieved by leaving the sections shortly in 96% ethanol, 70% ethanol, 50% ethanol and in aqua dest.

4.2.2 Mounting

To avoid air bubbles, drying out of the tissue and bleaching of the staining the right mounting medium needs to be chosen as a final step to conclude the whole labeling procedure.

When the staining was developed using DAB for detection the mounting medium of choice was Eukitt®. To use this medium the sections needed to be dehydrated in an ascending alcohol series (1x 50% ethanol, 1x 70% ethanol, 3x 96% ethanol and ether) and were mounted out of ether with cover slips.

In case of fast blue, fast red or double labelings Geltol was used as embedding medium. Sections were not dehydrated, but were covered out of water, since otherwise all the staining would have been washed away. The preparation of geltol is listed below.

Geltol: 60g liquid Geltol
24g Mowiol
mixed for 30 minutes up to 1h
+ 60ml aqua bidest.
incubation for 2h
+ 120ml Tris (0,2M pH = 8,5)
heated to 50°C while mixing the solution
when 50°C were reached still left for a 10 minute-incubation time
centrifugation at 5000G for 15 minutes
storage at -20°C

To finish the staining for confocal microscopy the sections were mounted with Gallate-Geltol. This medium was very similar to geltol. There was just 0,1M Gallate included in the medium (0,2g gallate in 10ml geltol), which needed to be mixed with geltol overnight before use.

4.3 Hämalaun-Eosin staining

4.3.1 Materials:

- Mayer’s Hematoxylin (Merck®)
Materials and Methods

- 0.5% HCl-alcohol
- Scott’s solution: 2g KHCO₃
  20g MgSO₄ x 7H₂O
  1000ml H₂O
- Eosin stock solution: 10g eosin
  100ml aqua dest
- Eosin ready-to-use solution: 2.5ml stock solution
  250ml aqua dest.
  12 drops glacial acidic acid

4.3.2 Method:

For HE staining the sections needed to be deparaffinized. Accordingly, the slides were incubated for 5 minutes in Mayer’s Hematoxylin, washed 3-4 times with H₂O and differentiated 5 times in HCl-alcohol. Next, washing two times with H₂O was performed, followed by incubation of the sections for 5 minutes in Scott’s solution and rinsing with water. Dyeing in eosin solution was done by a 3 minutes incubation time and to continue the method two times washing with water was performed. As a last step the slides were dehydrated using an ascending alcohol series and were mounted with cover slips using Eukitt® for embedding.

In the HE-staining the nucleic acids are colored in blue due to hematoxylin and collagen fibers and elastic fibers are dyed in pink due to eosin labeling.

4.4 Klüver Barrera – PAS

4.4.1 Materials:

- 0,1% luxol fast blue: 1g luxol fast blue
  1000ml 96% alcohol
- 0,1% hydrous lithiumcarbonate solution
- 70% ethanol
- 0,8% perjodic acid
- Schiff’s reagent
- sulfit washing solution
4.4.2 Method:

Klüver-Barrera staining was performed on deparaffinized sections, but rehydration was skipped. Slides were rinsed two times in 96% ethanol and were incubated in 0,1% luxol fast blue at 57°C overnight. After cooling the sections down to room temperature, they were rinsed in 96% ethanol, followed by washing with aqua dest. Then incubation in 0,1% hydrous lithiumcarbonate solution was performed for 5 minutes at room temperature. To continue, the staining was differentiated in 70% ethanol until the background staining was nearly gone and only the staining of the myelin sheath was visible. The PAS-reaction (perjodic acid Schiff stain) followed these few steps. The reaction was started with rinsing the slides with aqua dest, incubation for 10 minutes in 0,8% perjodic acid, washing with aqua dest. and incubation for 20 minutes in Schiff’s reagent. Next, the sections were put two times for two minutes into sulfit washing solution and then washed for 5-10 minutes with tap water. Finally, the sections were dehydrated using an ascending alcohol series and mounted with Eukitt®. The intention of the Klüver-Barrera – PAS staining is to make the myelin sheaths visible in blue and the grey matter in pink/red. In case of the viral encephalitis diseases being investigated here and in any other demyelinating diseases the demyelinated lesions get visible with this labeling.

4.5 Immunohistochemistry

In immunohistochemistry so called primary antibodies (listed in Table 2) were used to bind to specific targets, which can be visualized using different methods. In most of the stainings biotinylated secondary antibodies and avidin-peroxidase were used to detect the specific markers. If double staining for light microscopy was performed different detection methods were combined, such as the Alkaline Phosphatase (AP) system with the diazonium salt Fast Blue (FB) with diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC).

In case of confocal double labeling targets of investigation were tagged with different fluorescence dyes (Cy2, Cy3 and Cy5) for visualization.
### 4.6 Primary antibodies used in the study

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Antibody type</th>
<th>Target</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Rabbit (poly AB)</td>
<td>Immune Cells</td>
<td>steamer 60' citrate/EDTA 9.0</td>
<td>1:2000 with CSA</td>
<td>Neomarkers</td>
</tr>
<tr>
<td>CD8</td>
<td>Mouse (mAB)</td>
<td>Cytotoxic T-cells</td>
<td>steamer 60' EDTA 9.0</td>
<td>1:250</td>
<td>DAKO</td>
</tr>
<tr>
<td>GrB</td>
<td>Mouse (mAB)</td>
<td>Granzyme B</td>
<td>steamer 60' EDTA 9.0</td>
<td>1:50 or 1:1000 + CSA</td>
<td>Neomarkers</td>
</tr>
<tr>
<td>SV40</td>
<td>Rabbit (poly AB)</td>
<td>JC-Virus T-Antigen</td>
<td>steamer 60' citrate/EDTA 9.0</td>
<td>1:200 C or 1:500 E</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>SV40</td>
<td>Rabbit (poly AB)</td>
<td>JC-Virus T-Antigen</td>
<td>steamer 60' citrate</td>
<td>1:20000 with CSA or 1:5000</td>
<td>Aliquot Dr. Höftberger (6.9.2011)</td>
</tr>
<tr>
<td>SV40</td>
<td>Mouse (mAB)</td>
<td>JC-Virus T-Antigen</td>
<td>steamer 60' citrate</td>
<td>1:100</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Pab2003</td>
<td>Mouse (mAB)</td>
<td>JC-Virus VP1</td>
<td>steamer 90' EDTA 9.0</td>
<td>1:2000</td>
<td>Aliquot Frisque (27.03.06)</td>
</tr>
<tr>
<td>CMV</td>
<td>Mouse (mAB)</td>
<td>early antigen of CMV</td>
<td>steamer 60' EDTA 9.0</td>
<td>1:5000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>HSV</td>
<td>Rabbit (poly AB)</td>
<td>HSV type I and II</td>
<td>steamer 60' citrate</td>
<td>1:1000</td>
<td>DAKO</td>
</tr>
<tr>
<td>CM1</td>
<td>Rabbit (poly AB)</td>
<td>Caspase 3</td>
<td>steamer 60' citrate</td>
<td>1:50000 with CSA or 1:3000</td>
<td>Idun</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Rabbit (poly AB)</td>
<td>Caspase-6</td>
<td>steamer 60' citrate</td>
<td>1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>AIF</td>
<td>Rabbit (poly AB)</td>
<td>Apoptosis inducing factor</td>
<td>steamer 60' citrate</td>
<td>1:250</td>
<td>Chemicon</td>
</tr>
<tr>
<td>AIF</td>
<td>Sheep (polyAB)</td>
<td>Apoptosis inducing factor</td>
<td>steamer 60' citrate/EDTA 9.0</td>
<td>1:1000</td>
<td>R&amp;D systems</td>
</tr>
<tr>
<td>PAR</td>
<td>Mouse (mAB)</td>
<td>Poly (ADP-ribose)</td>
<td>no pretreatment</td>
<td>1:250</td>
<td>Aliquot Dr. Gallyas (16.10.08)</td>
</tr>
<tr>
<td>CAII</td>
<td>Sheep (polyAB)</td>
<td>Carbonic Anhydrase II</td>
<td>steamer 60' EDTA 9.0</td>
<td>1:1000</td>
<td>Binding site</td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Primary Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNPase</td>
<td>Mouse</td>
<td>2',3'-cyclicnucleotide-3'-phosphodiesterase</td>
<td>steamer 60' EDTA 9.0</td>
<td>SMI 91, Sternberger (25.2.05)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Mouse</td>
<td>Glial fibrillary acidic protein</td>
<td>steamer 60' citrate</td>
<td>Neomarkers</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit (poly)</td>
<td>Glial fibrillary acidic protein</td>
<td>steamer 60' EDTA 9.0</td>
<td>DAKO</td>
</tr>
</tbody>
</table>

Table 2 Primary antibodies used in the study

In the table the markers are listed according to the target they are binding to. The first antibodies (1-5) are specific for immune cells, the following markers detect infected cells (6-8). Accordingly, antibodies specific for targets involved in cell death are listed (9-12) followed by cell-type specific markers.

**Immune cell markers:**

**CD3:** CD3 is a co-molecule arranged around the t-cell receptor (TCR) and is characterized by the immunoreceptor tyrosin-based activation motif (ITAM) (Reth, 1989). Due to phosphorylation by cytoplasmic kinases (Iwashima et al., 1994) CD3 gets clustered and conformational changes are initiated. All this events are needed to induce TCR-signaling (Minguet et al., 2007). Since CD3 is expressed on all T-cells, it is commonly used as a marker for those cells (Bo et al., 2003).

**CD8:** CD8 is a transmembrane glycoprotein and serves as a co-receptor for the TCR. There are two possibilities how CD8 can be made up. One possible structure is the disulfide-linked α₂-homodimer and the other possible structure is a disulfide-linked αβ-heterodimer. CD8 shows in both conformations an immunoglobulin-variable like structure and also the same properties in the N-terminal extracellular domains (Leahy et al., 1992). CD8 is predominantly expressed on cytotoxic T-lymphocytes (CTL) and only rarely found on natural killer cells (NK-cells). The main function of CD8 is to specifically interact with MHC class I molecules (Swain, 1983).

**Granzyme B:** Granzyme B is one of the five serine proteases, so called granzymes, which is expressed in human (Chowdhury and Lieberman, 2008). The enzyme is released by CTL and NK-cells, if those cells interact with virus infected cells. In the infected cells granzyme B can induce cell death using two different pathways, one is the classical apoptosis pathway inducing the programmed cell death by cleavage of pro-caspase-3 to activated caspase-3. The other way is the promotion of mitochondrial permeabilization and cytochrome c release due to cleavage of BH3 interacting domain death agonist (BID). Accordingly, DNA fragmentation is induced. (Adrain et al., 2005; MacDonald et al., 1999; Martinvalet et al., 2008).
Materials and Methods

Virus markers:
SV40: The simian virus large T-antigen 40 is a multifunctional protein. It organizes the replication of the double-stranded viral genome and consequently the protein’s structure is optimized to exhibit this function (Meinke et al., 2011). The molecule shows three different functional domains, an N-terminal J-domain, an origin-binding domain (OBD) and a helicase domain (Pipas, 2009). The protein can assemble into single and double hexamers and according to its property it binds to specific DNA sequences on the origin of replication (ORI).

PAb2003: The antibody called PAb2003 is binding to early proteins expressed by the JC-virus (Munoz-Marmol et al., 2004).

HSV: The targets of the antibody are HSV-type I and type II.

CMV: CMV-antibody labels the virus infected cells through binding to an immediate early non-structural antigen (68-72kDa).

Markers for targets involved in cell death:
CM1: The so called CM1 antibody detects the enzyme caspase-3. Caspase-3 belongs to the family of cystein-aspartic acid proteases. Caspase-3 is usually present in the cytosol as an inactive form, namely pro-caspase-3 and only upon activation of initiator caspases (-2, -8, -10), which are acting more upstream cleavage and initiation of caspase-3 executioner activity can be induced. The direct consequence of caspase-3 activation is programmed cell death (apoptosis) (Olsson and Zhivotovsky, 2011).

Caspase-6: Caspase-6 is like Caspase-3 and -7 an executioner caspase. Like the other effector caspsases the immature zymogen form of the caspase needs to be cleaved by initiator caspsases to convert into the active conformation (apo-caspase). Caspase-6 (like the other executioner enzymes) is known to lead to degradation of critical proteins, nuclear and cytoskeletal proteins and consequently to cell death (Vaidya and Hardy, 2011). But it was also found that caspase-6 is involved in non-apoptotic processes like axonal pruning during development (Nikolaev et al., 2009), B-cell activation and B-cell differentiation (Watanabe et al., 2008).

AIF: Apoptosis inducing factor is a 67 kD flavin-adenine dinucleotide containing, NADPH depending oxidoreductase (Miramar et al., 2001). It is residing in the intermembrane space of mitochondria and is cleaved upon apoptotic insult (Otera et al., 2005), gets released and translocates to the nucleus, where it induces large-scale DNA-fragmentation in a caspase-independent manner (Cande et al., 2002).
**Materials and Methods**

**PAR:** Poly(ADP-ribose) is a negatively charged molecule that is synthesized by poly(ADP-ribose)-polymerase-1 out of nicotineamide adenine dinucleotide (NAD) (D’Amours et al., 1999). The molecule can selectively interact with target proteins that are involved in reactions such as DNA repair, DNA metabolism and can bind to PARP-1, its synthesizing molecule (Virag and Szabo, 2002). Additionally, PAR is thought to play a central role in the new cell death pathway parthanatos. Normally, PAR is remaining in the nucleus, just in parthanatos-inducing conditions it translocates and accumulates in the cytosol (Andrabi et al., 2006) (Andrabi et al., 2008).

**CNS-cell type specific markers:**

**CAII:** Carbonic anhydrase II is one of the 16 isoforms of human metalloenzymes (Saada et al., 2011). Its function is to catalyze the hydration of CO₂. This reaction is mediated by the transfer of a water proton bound to zinc(II) of CA and as a side reaction is also resulting in the generation of a zinc hydroxide species of the enzyme (Pastorekova et al., 2004). In the brain CAII is most dominantly localized to oligodendrocytes and can therefore be used as a detection marker for those cells. CAII is further present in the myelin and choroid plexus epithelium (Kida et al., 2006).

**CNPase:** 2’3’-cyclic nucleotide 3’-phosphodiesterase is specifically expressed in myelin and located to the cytoplasm of non-compact myelin. The enzyme catalyzes the phosphodiester hydrolysis reaction of 2’3’-cyclic nucleotides to 2’-nucleotides (Brunner et al., 1989) (Kursula, 2008).

**TPPP/p25:** Tubulin polymerization promoting protein is a member of the tubulin binding-proteins. TPPP/p25 is located to tubulin/microtubules. It is implicated in the stabilization of the microtubule network and in microtubule assembly in vitro (Vincze et al., 2006). In the brain TPPP/p25 is found in myelinating oligodendrocytes and neurons, but not in astrocytes (Cahoy et al., 2008). Further, TPPP/p25 is involved in oligogenesis, especially in the differentiation of oligodendrocytes (Lehotzky et al., 2010).

**GFAP:** Glial fibrillary acidic protein is a brain-specific intermediate filament expressed by astrocytes and Schwann cells. Like all intermediate filaments GFAP is involved in the cell shaping. However, it is also thought to be involved in calcification and chondrocyte differentiation due to its expression in elastic cartilage and cartilaginous differentiating tumors (Kepes and Perentes, 1988) (Webster et al., 1985).
4.7 Secondary antibodies used in the study

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
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Table 3 Secondary antibodies (biotinylated and coupled to AP) used for light microscopy

4.8 Secondary antibodies used for confocal fluorescence microscopy

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<th>Dilution</th>
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<td>Cy5</td>
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Table 4 Secondary antibodies used for confocal laser fluorescence microscopy specific for the various species and coupled to different fluorescent dyes

### 4.9 ABC-System using peroxidase activity for detection

#### 4.9.1 Materials:

- 0.02 % H₂O₂ – Merthanol: H₂O₂: 30% (Merck)
- EDTA (pH = 9 or pH = 8,5) (pretreatment-solution)
- Citrate-buffer (10mM) (pretreatment-solution)
- TBS (pH = 7,5) (wash buffer)
- PBS ready to use: PBS-stock (0,2M Phosphate buffer (Sörensen) diluted 1:4 with A.dest. (wash buffer)
- 10% FCS/Dako: fetal calve serum (Cambrex®)
  - DAKO® wash buffer (code S 3009) diluted 1:10
- primary antibodies and biotinylated secondary antibodies (see Table 2 and Table 3)
- avidin-HRP(Sigma-Aldrich®): 1:100 in 10%FCS/Dako
- biotinylated tyramine stock (CSA-stock): 6ml borate buffer (pH = 8,0) (Merck®)
  - 15ml sulfo-NHS-LCS-biotin (Pierce, Illinois, USA)
  - 4,5mg tyramin (Sigma-Aldrich®)
  - left for stirring overnight, filtered and stored in small aliquots at -20°C
- CSA used solution: 20µl CSA
  - 20ml PBS
  - 20µl H₂O₂
- avidin-HRP (1:200) in 10%FCS/Dako
- DAB: 1ml DAB (K3467 DAKO®) stock solution
  - 50ml PBS
  - 16,5 µl H₂O₂
- AEC: 1,15ml AEC (K3464 DAKO®) stock
50ml cold sodium-acetate-buffer (0.05M pH = 5)
25µl H₂O₂

4.9.2 Method:

All investigations were performed on paraffin-embedded tissue that was cut into 3-5µm thick sections. To start immunohistochemical stainings the sections were first deparaffinized, the endogenous peroxidase activity was blocked and rehydration was performed, as described earlier.

For antigen retrieval the deparaffinized sections were pretreated in EDTA (pH = 9 or pH = 8.5) or Citrate-buffer in a common house hold steamer (MultiGourmet FS20; Braun, Kronberg-Taunus, Germany) for one hour or 90 minutes. Depending on the used antibody the appropriate pretreatment solution and pretreatment-duration (60 minutes or 90 minutes) were chosen (see Table 2). After cooling down to room temperature the slides were washed 3-5 times with tris buffered saline (TBS) or phosphate buffered saline (PBS). Unspecific background signal was reduced by blocking with 10% FCS/Dako® for 20 minutes in a hybrid chamber at room temperature. The next step was to dilute the primary antibody for the target of interest in the right proportion (Table 2) in 10% FCS/Dako®. This application was performed overnight at 4°C. The slides were rinsed in TBS or PBS for 3-5 times after this incubation time and subsequently a biotinylated secondary antibody that was able to bind the primary antibody was applied, diluted in 10% FCS/Dako® (see Table 3) and left for incubation in a humid chamber on a slow shaker for one hour at room temperature. After washing the sections with TBS or PBS for 3-5 times, a one hour incubation period with avidin horse radish peroxidase (HRP) complex (1:100) was performed, followed by a washing step with buffer (TBS or PBS).

If necessary the labeling was amplified using biotinylated tyramine (King et al., 1997) (Bien et al., 2002), followed by washing and incubation with avidin-HRP (1:200) for 30 minutes at room temperature on a slow shaker.

To visualize peroxidase activity either diaminobenzidine (DAB) or 3-amino-9-ethyl-carbazole (AEC) could be applied. To stop the enzyme reaction the sections were rinsed with deionized H₂O for several times.

Counterstaining was performed with Mayer’s hematoxylin (10-20 seconds), followed by washing with H₂O, shortly rinsing in HCl-alcohol, washing with H₂O, blueing in Scott’s solution and dehydration in an ascending alcohol series (1x 50%, 1x 70%, 3x 96% and 1x ether). Finally, sections were mounted and were left for drying at room temperature for some hours before further examinations.
4.10 ABC-system using alkaline phosphatase activity for detection

4.10.1 Materials:

- avidin-AP: 1:100; Sigma-Aldrich®
- fast blue 50ml solution:
  6,25mg naphtol-AS-MX-phosphate
  + 312,5 µl DMF
  + 50ml warm Tris/HCl buffer (0,1M pH = 8,5)
  12,5mg fast blue BB salt mixed
  + 312,5µl 2N HCl
  + 312,5µl 4% NaNO₂
  → added to Tris/HCl-solution
  + 77µl 1M Levamizole
  incubation for 2 minutes at 37°C
  filtration

- All other materials (like wash buffer, pretreatment solution) were identical to those listed in the materials-part for ABC-system using peroxidase activity for detection.

4.10.2 Method:

Instead of using peroxidase activity as a detection method alkaline phosphatase (AP) activity can also be used to visualize the interesting targets. Generally, there was little difference between these two methods. The sections were deparaffinized, pretreated for antigen retrieval and primary antibodies as well as secondary antibodies were applied as mentioned above. The difference was that avidin alkaline phosphatase (AP) (1:100) was applied instead of avidin-HRP for one hour at room temperature on the slow shaker.

To visualize the labeling fast blue salt was used. Since development of the fast blue signal could take more time than DAB or AEC development, sections could be incubated in the fast blue solution up to 45 minutes at 37°C. As in the other staining methods the labeling reaction was stopped by washing the samples with water.

Counterstaining with Mayer’s hematoxylin can be performed, but in case of double labeling using SV40 as one of the primary antibodies, counterstaining was not part of the staining protocol due to a weak SV40-labeling and a possible confusion of the two (blue) signals.
Covering was done using Geltol as a mounting medium. To keep the sections free from air bubbles they were left in the fridge for drying overnight.

4.11 Double Labeling for Light Microscopy

4.11.1 Materials:

The materials for double stainings for light microscopy are all the same as in the single labeling for light microscopy (see in detail in 4.9.1 and 4.10.1).

4.11.2 Method:

Light microscopical double staining was performed as described earlier for ABC-system. Just some small changes were made. Pretreatment was chosen according to signal intensity and antibody efficiency in order to get the right targeting for both of the primary antibodies used. Then the optimal pretreatment was chosen, which was mainly depending on the antibody giving the weaker signal. Different primary antibodies of different species were simultaneously applied on the deparaffinized sections overnight at 4°C. Dilution of the antibody changed only for the primary antibody that was directly conjugated to a detection system to a less diluted concentration. After washing with TBS or PBS a second incubation with the primary antibody solution was performed for one hour at room temperature. This step was supposed to intensify the labeling. This was again followed by washing with buffer (TBS or PBS). The different secondary antibodies were applied simultaneously at room temperature. There was always one secondary antibody used directly conjugated to a certain detection method (like f. ex. α-mouse-AP) and one biotinylated secondary antibody. Depending on the use of secondary antibodies avidin-HRP or avidin-AP diluted 1:100 in 10%FCS/Dako was applied on the sections for one hour at room temperature.

To finish the staining alkaline phosphatase activity could be visualized with the diazonium salt fast blue or with fast red and peroxidase activity could be visualized either with DAB or AEC (described earlier). These reactions were performed after each other and depending on the target of interest and their signal intensity the right order for developing was chosen. To stop the detection reactions the slides were rinsed with H₂O and mounted with cover slips out of water with Geltol as the embedding medium.

All incubations at room temperature were performed on a slow shaker and in a humid chamber, just as incubation overnight at 4°C was done in a humid chamber, to keep the sections from drying out.
If there were double stainings performed using two primary antibodies coming from the same species the protocol was modified once more. In this case the antibodies were not applied simultaneously, but were incubated on the sections after each other. First antigen retrieval was performed as usual, followed by overnight incubation with the first primary antibody. Then application of the secondary antibody solution and the avidin solution were performed. According to the staining method used the proper avidin either coupled to horse radish peroxidase or alkaline phosphatase was chosen. Visualization of the first antibody was achieved mostly with diazonium salt fast blue. After stopping this labeling reaction the slides were once again kept in the household steamer (for 15-30 minutes) in the best suiting buffer (citrate or EDTA). This incubation was not necessarily performed to obtain a better signal of the second antibody, but was done to denature all the unbound staining components still remaining from the first reaction and therefore avoid cross reactions and reduce the background signal in the second staining reaction. All further steps were performed as mentioned above (incubation with primary antibody overnight, application of secondary antibody solution and avidin-solution each for one hour). In some cases MACH4 system was used (after overnight incubation with the primary antibody). The advantage of this system is that there is no need for using another biotinylated secondary antibody and therefore chances of cross reaction and background signal are reduced. To achieve a proper staining with this system the sections were incubated for 15 minutes first with MACH4-mouse probe, followed by washing and incubation with HRP-polymer (MACH4) for 15 minutes. If a primary antibody taken out of rabbit was used only application of HRP-polymer (MACH4) for 30 minutes was performed. As a last step the labeling was visualized by using 3,3’ diaminobenzidine tetrahydrochloride hydrate (DAB). Sometimes ammoniumnickelsulfate (1%) (1ml to 50ml DAB) and cobalt (II)-chloride (1%) (0,6ml to 50ml DAB) were added to the DAB solution to intensify the staining and enhance the contrast of the labeling.

4.12 Confocal Laser Fluorescence Microscopy

4.12.1 Materials:

- Dako-Diluent (code S2022)
- primary antibodies (see Table 2)
- 10% FCS/Dako®: FCS (Cambrex®)
  Dako®-wash buffer (S3006) diluted 1:10 with A.dest.
- secondary antibodies (biotinylated and conjugated to cyanine dyes) (see Table 3 and Table 4)
- streptavidin conjugated to fluorescent dye (Jackson ImmunoResearch) 1:75
Materials and Methods

- Dapi (Sigma-Aldrich®) 1:10000 in PBS
- TBS (pH = 7.5) or PBS as a wash buffer

4.12.2 Method:

Except for small variations fluorescence immunohistochemistry was performed very similar to double labeling for light microscopy with small variations. In case of a double staining for confocal microscopy blocking of the deparaffinized sections was performed with Dako-Diluent. Both primary antibodies of the two different species were used more concentrated than in single labeling IHC and were diluted in Dako-Diluent. Application was performed simultaneously at 4°C overnight. The slides were washed with TBS or PBS and incubation with primary antibody solution was repeated for one hour at room temperature. After washing with buffer (TBS or PBS), application of secondary antibody solution (see Table 4 for dilutions), containing one biotinylated secondary antibody and one directly fluorescence conjugated secondary antibody diluted in 10% FCS/Dako®, followed for one hour at room temperature on a slow shaker. The staining was continued by incubation with streptavidin conjugated to a fluorescence dye for one hour at room temperature, washing and counterstaining with 4′,6′-diamidino-2-phenylindole (Dapi) for 5 minutes at room temperature. Mounting of the slides with cover slips was done using gallate-geltol as a mounting medium.

In case of a triple labeling the three different antibodies of different species were applied simultaneously, as well as the secondary antibodies were applied together (as mentioned above). All other steps were performed identically.

During incubation times the sections were always kept in a light proof humid chamber to avoid drying of the samples and the dyes from bleaching.

Fluorescent preparations were examined using a confocal laser scan microscope (Leica SP5; Mannheim, Germany).

4.13 Quantitative analysis

In order to gain some quantitative information about the investigated sections, manual counting on the microscope was performed. Therefore an ocular grid was used. Most of the countings were done using the 20 times magnification objective to make sure that the cells showing the right properties were counted. With this magnification the grid has a length of 0,48mm and a total volume of 0,2304mm². Along with these grid values and the counted cell values the amount of cells present in 1mm² was calculated. If the area, in which the counting was performed, did not contain any cells showing the
properties under investigation, the area was enlarged and consequently other grid values for counting were used.

For statistical analysis IBM SPSS 18 was used. Since our data showed an uneven distribution and due to the low case number, we only performed nonparametric tests. In the comparison of the data a pairwise calculation of significant differences between two diseases was performed. This calculation was achieved using the Mann-Whitney-U-test. Generally p-values below 0.05 were considered to be significant.
5. Results

5.1 Basic pathological features in viral encephalitis

In this study paraffin-embedded PML samples of 8 different HIV+ patients were used for the experiments. Firstly, histochemical stainings with luxol fast blue for myelin sheaths for visualization and localization of the demyelinating lesions in the white matter of our PML samples were performed. The next step was the immunohistochemical labeling for the presence of virus infected cells with the SV40 antibody (performed by Dr. Höftberger on the AKH in Vienna), detecting the T-antigen of JC-virus. In this SV40 staining it could be observed that the infected oligodendrocytes were present on the edge of the lesions, whereas in the lesion center no infected oligodendrocytes could be detected. The fact that the infected cells were primarily oligodendrocytes was verified using a double labeling for SV40 T-antigen and CAII as a marker for oligodendrocytes. Bizarre shaped, virus containing astrocytes were found in the middle of the demyelinating lesion. These bizarre cells were astrocytes as seen by double staining for the JC-virus and GFAP an astrocyte marker (shown in Figure 6).

On the basis of the SV40 labeling, information on lesion size and amount of cells that are containing JC-virus was gained. Most of the virus infected oligodendrocytes were found in sections with many small lesions that were in close vicinity with each other and sometimes also connected to each other due to lesion spreading. In big lesions virus infected oligodendrocytes were found predominantly on the border whereas virus containing astrocytes were mainly detected in the lesion center. Virus expressing oligodendrocytes were usually characterized by the presence of a round swollen nucleus that was much bigger in size than the nucleus in non-infected oligodendrocytes. Generally, it was found that also the cytoplasm of these virus infected oligodendrocytes were enlarged compared to their usual appearance. This typical property could also be used for identification of infected oligodendrocytes in other stainings. Infected astrocytes could be identified due to their strikingly enlarged nucleus and swollen cytoplasm, which is very different from the usual astrocyte appearance.

An interesting additional feature that could be detected in the virus staining was that some of the cells labeled with the JC-antibody appeared to be degenerating. The cell body of these cells was blebbing, whereas their nucleus was shrinking and the chromatin was seen to become condensed. Astrocytes were seen to be enlarged and their cytoplasm was seen to be totally full with JC-virus, but astrocytes were not seen to degenerate. The results of the JC-virus stainings were fundamental for all our following experiments, since we gained knowledge about the special properties of each sample used.
Results

Figure 6 Labeling of SV40 infected cells for confocal laser microscopy (630x magnification used in all pictures): a shows a virus infected oligodendrocyte, whereas the virus marker was visualized with DyLight549 in red and CAII the oligodendrocytemarker was visualized with Cy5 seen here in blue; b shows a virus infected astrocyte (SV40 is seen in red (DyLight549) and GFAP is labeled in green (DyLight488)); c virus infected oligodendrocyte (double labeled with DyLight549 (SV40) and Cy5 (CAII) and an astrocyte that is not virus infected; d double labeled astrocytes for GFAP (DyLight488) and SV40 (DyLight549)

Typical properties seen in CMV encephalitis were that the infected cells were mainly present around ventricles or areas to which CSF had access. The infected cells which were mainly neurons and astrocytes appeared highly enlarged and showed viral inclusions in the nucleus. The typical “owl eye” shape was seen in many of the infected cells. This special feature was used in later stainings to identify the infected cells. Further microglia nodules were observed as well as necrotic lesion appeared in the in the brain parenchyma, resulting in cell loss.

In HSV encephalitis as well as in the CMV encephalitis necrotic lesions were observed. Additionally, to the necrotic lesions also hemorrhagic properties were observed in the lesions of the HSV encephalitis patients. HSV infected cells were not showing any special properties which would have facilitated identification. Therefore stainings for different cell type markers were performed in order to identify the underlying cell type. The results showed that mainly neurons, astrocytes and
Results

oligodendrocytes were infected with HSV. The virus was present in the nucleus as well as in the cytoplasm.

5.2 Inflammation in viral encephalitis

To gain more information about the role of inflammation in this viral encephalitis disease staining for CD3 (a general T-cell marker), CD8 (a marker for cytotoxic T-lymphocytes) and labeling for granzyme B (GrB), a protease released by cytotoxic T-lymphocytes (CTL) as defense mechanism, was performed.

Figure 7 Immune cells invading the brain parenchyma; a-c DAB stainings for CD3; a CD3 cells in PML tissue (40x magnification); b CD3 in CMV encephalitis is already showing appositions with the infected cells (100x magnification); c CD3 in HSV encephalitis (100x magnification); d-f DAB staining for CD8; d CD8 cells in PML (40x magnification); e CD8 in CMV encephalitis (100x magnification); f CD8 in HSV encephalitis (100x magnification); g-i DAB staining for GrB; g GrB positive cells in PML (250x magnification); h GrB cells being in apposition with CMV infected cells in CMV encephalitis (250x magnification); i GrB positive cells in HSV encephalitis (250x magnification)
Results

To compare the results of our investigations on the PML-samples and check for similarities in other viral encephalitis diseases, the experiments described above for PML were performed in the same way on samples taken from patients suffering from herpes simplex encephalitis (HSV) and cytomegalovirus encephalitis (CMV) (see Figure 7). After immune cell quantification (see Table 5) statistical comparison of the number of cells stained for CD3, CD8 and GrB in the three different diseases was performed.

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<th>CD3/mm²</th>
<th>CD8/mm²</th>
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</table>

Table 5 Quantification data from PML, HSV encephalitis and CMV encephalitis

First analysis of the amount of CD3⁺ T-lymphocytes present in the brain parenchyma in the 3 forms of encephalitis was performed. Comparison of the PML cases with the HSV encephalitis cases showed an insignificant difference (p = 0.057). When PML was compared with CMV encephalitis, the result
showed significant statistical difference ($p = 0.028$). Another significant difference was seen in the comparison of HSV encephalitis with CMV encephalitis ($p = 0.007$). Next, the data for the CD8$^+$ T-lymphocytes, which was set in relation to the amount of CD3$^+$ cells, was compared between the three diseases. When checking for significant differences between PML and the other encephalitides we found again no difference with HSV ($p = 0.380$), but a difference with CMV encephalitis ($p = 0.011$). The results for the comparison of HSV encephalitis with CMV encephalitis revealed an insignificant result ($p = 0.122$). As a last step we also compared the data for the presence of GrB in relation to the amount of CD3$^+$ T-cells. Interestingly the detailed comparison of the three diseases indicated no significant difference between PML and HSV and PML and CMV as well as between HSV and CMV found (PML vs HSV: $p = 0.661$; PML vs CMV: $p = 0.132$; HSV vs. CMV: $p = 0.223$) (see Figure 8).

![Figure 8](image)

Figure 8 a amount of CD3/mm$^2$, CD8/mm$^2$ and GrB/mm$^2$ arranged in groups for each disease, the clamps indicate the significant differences obtained during comparison; b amount of CD8 (%) and GrB (%) set in relation to the amount of CD3 positive cells present in the brain parenchyma

In all three encephalitides it could be shown that viral infection resulted in recruitment of immune cells into the brain parenchyma, especially to sites of demyelinating lesions. In a double staining it could be visualized that CD8$^+$ cytotoxic T-cells and JC-virus containing cells were present in the same area, further it was observed in another staining that 29% of those CD8$^+$cytotoxic T-cells were containing GrB in PML. Double stainings using GrB and SV40 as markers verified that GrB$^+$ cells could be found in the same areas as the infected cells. Generally, these results are indicative for an involvement of the immune system in initiation of cell death in virus infected cells. However, since close contact of CTLs with their targets is absolutely needed, the appositions (single or multiple) of GrB containing cells with JC-infected cells were specifically analyzed. This analysis revealed that close apposition of SV40 positive cells with GrB containing cells was absent in the brain parenchyma.
Results

of PML-patients. GrB positive cells were sometimes relatively close to JC-virus containing cells, however there has not been seen a direct contact of these cells. Quantification of the numbers of infected cells with single or multiple appositions of GrB$^+$ cells in PML patients in comparison with HSV encephalitis and CMV encephalitis confirmed that appositions in PML were very rare. The comparison of PML with HSV encephalitis and CMV encephalitis revealed that the latter diseases had more appositions (see Figure 10).

Figure 9 Double labelings for GrB (labeled with DAB in brown) and the different virus markers; a labeling for SV40 with fast blue and GrB, showing that those cells are located in the same area, but no apposition was detectable (400x magnification); b multiple appositions of GrB positive cells towards the CMV infected cell (labeled with fast red), polarization of GrB towards the CMV positive cell can be seen and is indicated by the arrow head (400x magnification); c labeling of HSV infected cell (with fast red) and GrB, whereas the polarization of the cytotoxic granule is indicated by the arrow head (400x magnification)

The biggest difference was seen when comparing PML with CMV encephalitis. In this case statistical significance was detected (p=0,014), unlike the comparison of PML and HSV encephalitis which did not show significance (p=0,879). For HSV encephalitis and CMV encephalitis another insignificant result was obtained (p = 0,122) (shown in Figure 10). Moreover, unlike in PML, in CMV encephalitis it was even possible to detect multiple appositions, which means that multiple GrB$^+$ cells are in contact with one single CMV positive cell. In the comparison of the data for multiple appositions between the three diseases a statistically significant result was seen between PML and CMV encephalitis (p=0,008), but not for PML with HSV encephalitis (p = 0,642) and CMV with HSV (p = 0,110) (shown in Figure 10). Another step in cytotoxic killing of target cells by cytotoxic T cells is polarization of the cytoplasm and directing the GrB granules towards the target cell. Such GrB polarization was described in in vitro cytotoxicity and was seen to highly augment the cytotoxic capacity of those granules (Wagner et al., 1993). In addition to the above-mentioned quantitative findings, we could observe such polarisations of GrB towards the virus-infected cell in CMV and HSV encephalitis but not in PML encephalitis (Figure 9).
It has been shown in the past that infiltrating lymphocytes in brain of acute disseminated encephalomyelitis are eliminated by apoptosis (Bauer et al., 1999). During the examination of cytotoxic T cells in the brain presence of GrB$^+$ cells with clearly condensed nuclei indicative for apoptosis was noticed (Figure 11). Quantitative analysis of such apoptotic GrB$^+$ cells surprisingly showed that in PML these numbers were as high as 25%, whereas in HSV and CMV encephalitides these numbers were respectively lower and accounting for 4.8% and 4.8% (shown in Figure 12).
Results

Figure 11 ABC staining for GrB; a normal appearing GrB carrying CTL (400x magnification); b apoptotic GrB containing CTL showing a condensed nucleus (400x magnification)

Figure 12 Amount of apoptotic GrB cells counted in the brain parenchyma of the different viral encephalitides; clamps indicate the statistical differences calculated for the different diseases; it can be seen that there were much more apoptotic GrB positive cells found in PML compared to HSV encephalitis and CMV encephalitis and that statistical difference was obtained in the comparison of PML to the other two diseases.

In order to gain more information about the underlying mechanisms in the different viral encephalitis diseases the influence of executive caspases, which can be activated due to immune response and release of GrB, was under investigation investigated next.
5.3 The role of caspases

Since caspases, primarily caspase-3, can be activated by granzyme B during the defense mechanism and because of their role as the key enzymes in apoptosis, they might exhibit a central function in the course of oligodendrocyte cell death in PML. Accordingly, labeling for activated caspase-3 and caspase-6, two of the main executioner caspases in apoptosis, were performed. In apoptototic cells, caspase-3 is found in the cytoplasm (Bradl et al., 1999) (Bien et al., 2002) (Bauer et al., 2007). Inactive caspase-6 is present in mitochondria and thus shows a granular staining. During apoptosis caspase-6 diffuses from the mitochondria and can be found as a diffuse cytoplasmatic staining. The results of these stainings showed that caspase-3 and caspase-6 were expressed in their inactive conformation, but were sometimes present in an upregulated manner in the virus infected cells in our PML-samples. None of the JC virus infected oligodendrocytes contained activated caspase-3 or caspase-6 (see Figure 13). Still, we observed that in our samples (mainly on the edge of lesions) some cells showed a typical apoptotic nucleus. This led to the conclusion that initiation of cell death resulting in tissue damage and presence of cells with condensed nuclei needs to be induced in another way than T-lymphocyte activation, GrB release and caspase-activation in PML.

To compare the observed findings in the PML-cases with the HSV encephalitis and CMV encephalitis cases we also performed the stainings for caspase-3 and caspase-6 expression in those samples. The results of the caspase labelings in the other two encephalitides revealed that their expression was up regulated like in PML. The search for activated caspase-3 and activated caspase-6 in HSV and CMV was successfully concluded since cells containing the activated enzymes could be found. To confirm that the activated caspases were present in virus-infected cells double stainings for activated caspase-3 and the particular virus marker were performed. In the CMV encephalitis we could nicely visualize a colocalization of the of the virus marker with caspase-3 or cytoplasmatic caspase-6. This was also seen in the HSV cases albeit in less high numbers. Furthermore, in both the HSV encephalitis and in the CMV encephalitis we could show that these caspase-3 or caspase-6 positive cells contained a condensed nuclei typical for apoptosis (see Figure 13).
Figure 13 Single labelings for caspase-3 (first row) and caspase-6 (second row) and double labeling for virus markers with caspase-3 (third row): a shows inactive caspase-3 in the PML-sample (indicated with an arrow) (400x magnification); b activated caspase-3 (marked with an arrow) that is very darkly stained and widely distributed in the cytoplasm can be seen (400x magnification); c HSV encephalitis sample showing an astrocyte positive for activated caspase-3 (400x magnification); d activated caspase-3 in HSV encephalitis whereas this cell is already showing a condensed, apoptotic nucleus (marked with an arrow) (400x magnification); e staining for caspase-6 which is present in an upregulated manner, but though inactive and still granular (cells containing inactivated caspase-6 are marked with an arrow) (400x magnification); f caspase-6 present in its inactive conformation (granular staining) as well as the active conformation (distributed in the cytosol), cells containing caspase-6 in its active conformation are pointed out by an arrow head (400x magnification); g inactive, but upregulated caspase-6 as well as clearly apoptotic cells (marked with an arrow) with their nucleus surrounded by activated caspase-6 can be seen (400x magnification); h double staining for SV40 (in blue) and caspase-3 (red) (marked with an arrow) whereas no double labeled cells were detected (400x magnification); i confocal laser microscopy stained for CMV (green (DyLight488)), caspase-3 (red (DyLight549)) and counterstained with DAPI (blue) showing cells being double labeled for the virus marker and caspase-3 (630x magnification); j-k double labeled cell with HSV and caspase-3, whereas the double labeled cell in picture k is indicated by an arrow (400x magnification)

Summarised, these findings suggest that an immune response resulting in the activation of caspases induces apoptosis in HSV encephalitis and CMV encephalitis but that the situation for initiation of oligodendrocyte death is different in PML.
5.4 Cell death in PML goes via Parthanatos

According to the data shown above, the underlying cause for oligodendrocyte death in PML was very unlikely to be depending on the activation of CD8\(^+\) T-lymphocytes, release of GrB and initiation of apoptosis via activation of the caspase-cascade. Consequently, it was searched for another possible mechanism which was giving a better explanation for demyelination due to oligodendrocyte death in PML and which was also matching with the results described above. The in the introduction described form of cell death parthanatos was a likely candidate for the cell death in PML. Since poly-(ADP-ribose) (PAR) and apoptosis inducing factor (AIF) were the key molecules in the parthanatos pathway, stainings for these two molecules were performed (Andrabi et al., 2008) (Wang et al., 2011). In the immunohistochemical labelings using the PML-samples we could nicely visualize that in the JC-virus infected oligodendrocytes PAR, was not only located in the nucleus, but was often also seen to translocate to the cytosol. Triple staining with SV40, PAR and CAII confirmed that mainly virus infected oligodendrocytes were containing PAR in the cytosol. Further we observed that AIF was not totally restricted to the mitochondria, as seen under normal conditions, but was also translocated out of the mitochondria to the nucleus where it was able to induce DNA-fragmentation. To get better evidence for these results double and triple stainings for confocal microscopy were performed, like SV40, AIF and PAR or AIF, PAR and CAII (shown in Figure 14).

These labelings further proofed that in JC virus-containing oligodendrocytes PAR could be seen in the cytosol and AIF was colocalized with the virus staining in the nucleus. These observations support the theory that JC-virus-infection of oligodendrocytes initiates a process which leads to accumulation of PAR in the cytosol, translocation of AIF to the nucleus and as a last consequence to cell death.
Results

Figure 14 Stainings for parthanatos markers (AIF and PAR) in PML; a PAR (stained with DAB) is usually seen to be present in the nucleus, but during cell death initiation is accumulating in the cytosol (250x magnification); b PAR is widely distributed in the cytosol (400x magnification); c AIF (labeled with DAB) is found in the mitochondria showing a granular staining, but during parthanatos is translocated to the nucleus (250x magnification); d AIF found in the nucleus (400x magnification); e-f confocal laser microscopy used to label the JC virus (in blue with Cy5) located in the nucleus, PAR (with DyLight488 in green) located in the cytosol of the virus infected cell and AIF (with DyLight549 in red) also found in the nucleus, overlay of the stainings in the nucleus result in a yellowish color (630x magnification); g confocal laser microscopy for visualization of SV40 (with DyLight549 in red), PAR (with DyLight488 in green) and CAII (with Cy5 in blue) to verify that oligodendrocytes are the virus infected cells dying through parthanatos (630x magnification)

To further gain clear evidence that there were different mechanisms activated in the different viral encephalitis diseases single labelings for PAR and AIF were performed on the HSV encephalitis samples and CMV encephalitis samples. Surprisingly, it was found that PAR was present in the cytosol of some cells in HSV encephalitis. AIF, on the other hand could not be detected in the nucleus of any of the HSV encephalitis specimens. In the CMV encephalitis cases PAR could not be found in the cytosol. Also, AIF could not be seen to be translocated to the nucleus in the virus containing cells. To confirm this, we also performed triple stainings on those sections, using antibodies against the specific virus together with AIF and PAR. Again, we never found a translocation of PAR out of the nucleus into the cytosol and AIF out of the mitochondria into the nucleus in the virus infected cells (shown in Figure 15).
Figure 15 Confocal laser microscopy used to visualize parthanatos markers in CMV encephalitis and HSV encephalitis; a PAR (marked with DyLight488 in green) is mainly restricted to the nucleus and AIF (labeled with DyLight549 in red) is only seen as a granular staining only located in the mitochondria in CMV infected cells, DAPI staining is seen in blue and virus infected cells were identified due to their “owl eye” shape (630x magnification); b no indications for parthanatos can be found in HSV infected cells (labeled with Cy5 in blue), PAR (marked with DyLight488 in green) and AIF (stained with DyLight549 in red), since PAR was remaining in the nucleus and AIF was present in the mitochondria (630x magnification)

These findings strongly suggest that, unlike in PML, parthanatos is not the underlying cell death pathway in these two viral encephalitides
6. Discussion

Demyelination and oligodendrocyte loss is a central feature occurring during the disease course of PML (Astrom et al., 1958). Though being this important, it has never been established which mechanism is actually leading to death of virus infected oligodendrocytes and therefore causing the demyelinating lesions in the white matter. Oligodendrocyte loss could be the result of various mechanisms. First, it might be a direct result from the virus infection. After infection the virus uses the oligodendrocytes system to create new virus particles. During this phase protein synthesis and energy metabolism are constantly at high levels. Since viral replication and maturation is tightly connected with the function of the endoplasmatic reticulum (ER) this organelle senses changes naturally occurring in infected cells. A high protein traffic through the ER as well as misfolded or unfolded protein release due to the viral infection, might initiate ER stress probably leading to apoptosis (He, 2006). It might be that this overload finally leads to internal damage and death of the infected cells.

Secondly, inflammation is abundant in the brain of PML-patients, but a link between the initiation of the immune response and death of oligodendrocytes has not described. Generally it is assumed that cytotoxic T cells kill virus infected cells. Cytotoxic T cells can kill target cells in a number of ways, whereas two groups of mechanisms are distinguished. First group is involving death receptors with appropriate ligand binding and the second pathway is a granule-dependent. Generally death receptor killing can be either induced by TNF-TNFR interaction or by interaction of Fas and its ligand FasL (CD95) leading to downstream signaling cascade activation. Granule dependent killing on the other hand involves release of perforin towards the target cell membrane in order to make holes in the membrane. This consequently enables GrB to enter the cell, where this protease is capable of initiating caspase-dependent apoptosis (Russell and Ley, 2002). To find out if the cytotoxic T cells are really involved in the pathogenesis of PML, presence of CD3+ , CD8+ and GrB containing cells were analyzed first. The results showed that there are activated cytotoxic T-cells in the brain parenchyma. This finding was already described in a paper published by Wüthrich et al.. In this paper results are shown that CD8+ cytotoxic T-cells are located in the brain parenchyma and it is also claimed that appositions of CTLs and SV40 positive cells are found. The group interpreted these observations as an indication of cytotoxic T cell involvement in oligodendrocyte death (Wuthrich et al., 2006). However there were no experiments performed in this paper linking specific cytotoxic mechanisms directly to oligodendrocyte death, for instance it was not checked if cytotoxic granules were present in those CTLs being in apposition with virus-infected cells and therefore create the possibility that GrB release is responsible for oligodendrocyte apoptosis. Here the presence of GrB in the samples was counted and it was found that around 29% of CD8+ cells in the brain parenchyma carried GrB. In the double labeling it could also be shown that virus infected cells and GrB positive cells were present in the same area. However, although being located close to each other the search for appositions was not
successful. It was not possible to find any appositions of JC-virus containing cells with cells carrying cytotoxic granules.

In the comparison of PML with HSV encephalitis and CMV encephalitis differences could be observed. Unlike the findings in PML, it was possible to find single appositions of GrB containing cells with the specific virus present in both HSV encephalitis as well as CMV encephalitis. However in CMV encephalitis a much higher frequency of single appositions could be detected and even a high frequency of multiple appositions of GrB$^+$ cells to infected target cells was observed. This already manifested the idea that cytotoxic T-cells are involved in killing virus infected cells in CMV encephalitis. In HSV encephalitis on the other hand the situation was not this clear, but still there was a trend seen that activation of cytotoxic T-lymphocytes and GrB release plays a more crucial role than in PML.

At the present time it is unclear why in PML cytotoxic T cells do not seem to kill the infected cells by GrB release. A possible explanation could be that the infected cells are not detected by the cytotoxic T cells. In order to enable virus detection infected cells have to present viral proteins by MHC class I molecules (Adhya and Basu, 2010). Accordingly it could be estimated that JC-infected oligodendrocytes do not express these MHC class I molecules. Surprisingly, however, these infected cells revealed strong staining with HC10, an antibody recognizing the heavy part of the MHC class I molecule (Stam et al., 1990). However, viruses evolved various other mechanisms to escape the cytotoxic T-cell response. Possibilities to inhibit this response are to block the antigen processing and to interfere in the antigen transporter activity (Farrell and vis-Poynter, 1998). Consequences of these events are that no antigen is presented by the MHC class I, so no activation of cytotoxic T-cells is achieved and the virus can successfully escape cytotoxic T-cell killing. Other immune evasive strategies involve blocking of interferon-dependent signaling pathways (Smith et al., 1997) or changes in the chemokine or cytokine production in order to promote virus replication (Lalani et al., 2000) (Tortorella et al., 2000). Such mechanisms have not been described in PML.

Due to all these results the idea was formed that there was a difference in the involvement of the cytotoxic T cells between the three different viral encephalitis diseases. Since it was observed that 25% of GrB positive cells were apoptotic in PML the frequency of apoptotic GrB cells in the HSV encephalitis and CMV encephalitis was counted as well. The incidence rate for apoptotic GrB cells in these diseases was dramatically lower than in PML. In the HSV encephalitis as well as CMV encephalitis the same level of apoptotic GrB cells was observed. How apoptosis is induced in GrB carrying cells in PML is not known, but one could speculate that the JC virus evolved this strategy to escape cytotoxic T-cell killing. Inducing, apoptosis in GrB cells would be beneficial for the JC virus since those cells cannot exhibit their original function of GrB release into the target cell. Accordingly, the virus can further replicate and multiply. Of course these results were further enforcing the idea of different underlying mechanisms in the different diseases with a different role of the immune system.
Cytotoxic T-cell activation and GrB release can induce apoptosis. Therefore the apoptotic signaling cascade plays a major role. Generally, apoptosis can be induced by various triggers and the major enzymes in this pathway are caspases. The caspases can be divided into two groups; the initiator caspases, of which caspase-8, -9 and -10 are the most prominent ones and the executioner caspases. Caspase-3, -6 and -7 contribute to the last group. If apoptosis is induced, the initiator caspases are cleaved, transform to their active conformation and further activate executioner caspases. These enzymes can then cleave target proteins and induce apoptotic cell death (Fan et al., 2005). To check the effects of GrB release effort was put into finding out whether there were activated caspases present in the tissue. Results of caspase-3 labeling as well as the staining for caspase-6, showed that in PML we could not find any evidence that caspase-mediated apoptosis was really induced in the virus positive oligodendrocytes. Interestingly, in the past two papers have dealt with the topic of oligodendrocyte death, but the from these two publications are rather controversial. Accordingly, a convincing explanation for oligodendrocyte death has not yet been found. In one of those papers the authors claim, that apoptosis is the underlying mechanism leading to death of virus infected oligodendrocytes. As proof for this theory double staining of JC virus containing cells together with a TUNEL-staining is given (Richardson-Burns et al., 2002). However, the TUNEL labeling only recognizes DNA-double strand breaks which are occurring in both apoptotic as well as necrotic cells (Perry et al., ). As such the TUNEL staining is not giving any information on the pathway leading to cell death. In addition, the virus replication induces DNA strand breaks themselves and due to this also non-dying virus infected cells are recognized by TUNEL. In the second paper it is described that due to the virus infection survivin, an anti-apoptotic protein, is newly expressed in the virus containing cells and therefore protects those cells of getting killed due to initiation of apoptosis (Pina-Oviedo et al., 2007). Although this mechanism may extend the life of infected oligodendrocytes, the enormous loss of oligodendrocytes and severe demyelination show that eventually large numbers of these oligodendrocytes undergo cell death. Since the authors of these two papers hold an opposite view on the topic and since the obtained data showed no evidence that caspase-mediated apoptosis, initiated by cytotoxic T cells is involved in the death of oligodendrocytes, it was suggested that another mechanism might be involved. A recent paper from our group (Veto et al., 2010) shows that in multiple sclerosis (MS) pattern III lesions oligodendrocyte death and demyelination is induced due to overactivation of poly(ADP-ribose) polymerase 1 (PARP-1) and translocation of apoptosis inducing factor (AIF) into the nucleus. It is claimed that due to these events caspase-dependent apoptosis is prohibited and that cell death is initiated due to AIF accumulation in the nucleus of oligodendrocytes and therefore induces a caspase-independent cell death pathway (Veto et al., 2010). The results of this paper were very interesting and give a new explanation for oligodendrocyte death. Therefore it was checked whether similar events could be observed in the PML-samples. Staining for PAR, the side product of PARP-1, showed that this molecule, that is usually restricted to the nucleus, was widely distributed in the cytosol of the virus-infected oligodendrocytes. Furthermore, it was seen that AIF was
not restricted to the mitochondria, but was also present in the nucleus. This translocation of AIF to the nucleus can induce DNA-fragmentation and is able to lead to cell death. These events described here and detected in the PML-samples are the key events of so-called parthanatos. This apoptotic pathway is generally said to be independent of caspases and translocation of AIF into the nucleus is handled as the commitment point. Nevertheless, at a certain, rather late time point, also caspases get activated, but this activation is said to occur in such a late stage, that cell death is not dependent on the function of caspases anymore (Yu et al., 2006).

Of course all these observations were compared between the PML-cases and HSV encephalitis and CMV encephalitis cases. The first difference that was found in the comparison of the viral encephalitis diseases was that in the CMV cases a very clear double labeling of virus infected cells together with activated caspase-3 was present. In addition, we could show a release of caspase-6 from the mitochondria into the cytosol. This already made it rather clear that cell death in the CMV-infected cells is most likely induced due to caspase-mediated apoptosis. Analysis of the presence of PAR in the cytosol and AIF in the nucleus of the virus infected cells showed that infected cells did not have these properties. This, and the presence of multiple GrB⁺ cytotoxic T cells in close apposition to these infected cells strongly suggests that in CMV encephalitis, unlike in PML, cytotoxicity by T cells is the central mechanism behind the pathogenesis of this disease. Evaluation of data coming from the HSV encephalitis diseases, showed that such a clear difference as seen in the comparison of CMV encephalitis to PML is not present. Presence of activated caspase was observed in the HSV-samples, but in the double labeling for the virus and caspase-3 the frequency for these double labeled cells was definitely lower than in the CMV-cases. A possible explanation might be that at the time caspase-3 activity reaches detectable levels by immunohistochemistry all the viral proteins are used as substrates and for the caspases and therefore become undetectable. Alternatively, other mechanisms are involved in the pathogenesis of HSV-encephalitis. The pathways leading to cell death however have to be different from parthanatos, since the HSV cases were naturally checked for the key events. Interestingly, cells containing cytosolic PAR were sometimes detected, but the commitment event of AIF translocation towards the nucleus was never observed. Therefore, it was not possible to find cells containing the HSV virus, PAR in the cytosol and AIF in the nucleus at the same time. Additionally, those cells containing PAR in the cytosol were mainly located in an area of necrosis, so this cytoplasmic localization might result from events occurring in those cells due to a deathly insult leading to a necrotic form of cell death not involving the PAR/AIF pathway.

To summarize, the results of this study give a convincing explanation on how cell death in oligodendrocytes in PML is initiated. This could therefore lead to an end of the discussion if apoptosis is occurring in the JC virus infected oligodendrocytes. Whereas in PML encephalitis, T cell cytotoxicity is not responsible for the death of infected cells, in CMV encephalitis indeed results strongly suggest that CD8⁺ T cells are involved. In HSV encephalitis moderate numbers of appositions
of cytotoxic T cells were found as well as low numbers of caspase-3 positive infected cells, suggesting that T cell cytotoxicity plays a role to a certain extend. Still further information needs to be gained on cell death in HSV encephalitis in order to be able to interfere in this mechanism to be able to help patients to a better outcome. Since it seems to be pretty clear that in CMV encephalitis cell death is resulting from apoptosis, possibilities in prevention of apoptosis should form a central topic in research. Regarding PML encephalitis research, future efforts should be put in gaining information on the exact events happening during parthanatos. The central question here should be if there is a possible chance to interfere in this pathway in order to stop oligodendrocytes from being killed. Naturally, this knowledge should then be used for therapy development to improve treatment of this disease.
### 7. Abbreviations used

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<td>AB</td>
<td>antibody</td>
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<td>ABC</td>
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<td>natural killer cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PML</td>
<td>progressive multifocal leukoencephalopathy</td>
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<tr>
<td>SOCC</td>
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<td>viral protein 1</td>
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8. Acknowledgments

First, I want to thank my supervisor Jan Bauer for giving me the chance to do my diploma thesis in his lab. I had the opportunity to collect knowledge about different techniques and he was always sharing his knowledge with me. I want to thank him for his patience and his guidance through this thesis.

I want to acknowledge Marianne Leisser, Ulrike Köck and Angela Kury for their technical assistance and helping hands.

Further, I am really grateful that I had such an understanding group working with me. It was comforting to know that there was always someone who could help and give useful advises. Special thanks to Conny, Mesi, Maya, Isa, Simon and Lukas.

I also want to thank my friends that were very understanding during this time. Here want to express special thanks to Vali, who was always listening to me when I needed to talk and who was always giving me motivation.

Further, I want to thank my family, especially my parents, Gerlinde and Peter, and my brother, Berni, who were always there for me and supporting me. Thank you for always believing in me and encouraging me.

Finally, I want thank Simon for believing in me, for cheering me up after a bad working day and for always being there for me.
9. Reference List


10. Attachments

10.1 Curriculum vitae

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