THEILER'S VIRUS-INDUCED APOPTOSIS IN CEREBROVASCULAR ENDOTHELIAL CELLS

A Dissertation

by

MAMATHA SOMANATH NAYAK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

May 2003

Major Subject: Microbiology

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ABSTRACT

Theiler's Virus-Induced Apoptosis in Cerebrovascular Endothelial Cells.

(May 2003)

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Theiler's murine encephalomyelitis virus (TMEV) is classified as a cardiovirus in the *Picornaviridae* family. An enteric virus, TMEV spreads within the mouse population by the fecal-oral route. The neurovirulent GDVII strain of Theiler's virus causes a fatal encephalitis in all strains of mice following intra-cranial infection of the virus. Persistent BeAn strain of Theiler's virus causes a demyelinating disease in susceptible strains of mice, which is similar to the human disease - Multiple Sclerosis (MS). Although a well-recognized model for MS, the route of entry of the virus into the central nervous system (CNS) following natural infection has not been well understood. One of the proposed portals of entry includes the blood-brain barrier (BBB). This report indicates the ability of both the neurovirulent and the persistent strains of Theiler's virus to induce apoptosis in the functional units of the BBB – the cerebrovascular endothelial cells (CVE) both *in vitro* and *in vivo*. Induction of apoptosis in CVE was demonstrated by annexin staining, electron microscopy, DNA fragmentation assay, Hoechst staining and by caspase-3 staining. Corresponding to results by other authors, GDVII is a

stronger inducer of apoptosis in CVE compared to BeAn. Induction of apoptosis is dependent on the MOI of the virus. UV-inactivated virus is not capable of inducing apoptosis and induction of apoptosis appears to be an internal event not requiring activation of death receptors. Determining the pathway of induction of apoptosis by TMEV in CVE indicated the involvement of a Ca2+ dependent pathway for apoptosis – the calpain pathway. Involvement of calpain in apoptosis has been reported in MS. Induction of apoptosis in CVE *in vivo* was also demonstrated following the intra-peritoneal inoculation of Theiler's virus. Induction of apoptosis in CVE following Theiler's virus infection could lead to a breach of the BBB and entry of inflammatory cells as well as virus into the central nervous system. This finding could aid understanding the neuropathogenesis of Theiler's virus.

DEDICATION

"If you can dream it, you can do it"

- *Walt Disney*

To my Parents, for believing in my dreams and to Arun, for sharing my dreams.

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This dissertation is by no means the effort of just one person. Many have contributed to it, some through helping me out with my experiments and others by an encouraging word or gesture. I would like to take this opportunity to express my gratitude to all those who helped me in this endeavor.

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CHAPTER I

INTRODUCTION

MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is the most common demyelinating disease of the nervous system. In the US approximately 250,000 individuals suffer from MS, with 10,000 new cases reported each year (88). MS is an inflammatory disease of the central nervous system (CNS) white matter characterized by demyelination, focal T cell and macrophage infiltration, axonal injury and loss of neurological function. The pathological features consist of white matter (WM) plaques characterized by primary demyelination and death of oligodendrocytes within the center of the lesion. Early in the disease process, the blood brain barrier (BBB) is damaged and perivascular inflammatory cells enter the CNS and destroy the WM. Different types of MS exist and the disease is usually classified on the basis of the clinical course of the disease. The five main types of MS are (141):

1. Benign: This occurs in 10-15% of patients where the disease does not develop into the progressive form of the disease. The symptoms are mild to moderate and it does not lead to permanent disability.

This dissertation follows the style and format of Journal of Virology.

2. Relapsing-remitting: About 85% of people with MS begin with this form. It is characterized by one or two flare-ups every 1-3 years and this is followed by periods of remission. Symptoms worsen with each flare-up.
3. Primary progressive: This occurs in 10% of patients. From the first appearance of symptoms, the neurological functions deteriorate without periods of remission.
4. Secondary progressive: Following years of having relapsing-remitting, about half of the patients will enter a stage of continuous deterioration.
5. Progressive relapsing: This is essentially primary progressive MS with new episodes worsening the existing ones. This is a very rare form of MS and occurs in less than 5% of the cases.

MS usually manifests between the ages of 20 and 40 and affects women twice as often as men (121). The etiology of MS is largely unknown. A number of risk factors have been associated with susceptibility to MS. These include: race, sex, age, ethnicity, weather conditions, diet, socio-economic status, family history, migration and genetic factors. Among genetic factors, HLA class II genes exert an influence with HLA DR2 carrying a 4-fold relative risk (7). A further role for genetics is implied in the findings that in monozygotic twins there is an increased incidence of MS over dizygotic twins (53). But genetics alone is not sufficient to predispose to MS. There appears to be a complex interplay of more than one risk factor. There is increasing evidence for the role of viruses in the development of this disease (6, 15). MS outbreaks have been reported

in places such as Faroe Islands in 1950's following arrival of British troops, strongly indicating the involvement of an infectious agent (122). Studies have also suggested that migration to and from high-risk areas influences the development of the disease (120). There is also epidemiological evidence of increased risk of MS in persons with a history of childhood exposure to infectious agents (99). In addition, relapses in MS appear to occur following respiratory (54, 181) and gastro-intestinal viral infections (9). The most convincing evidence for the role of viruses in the disease process of MS comes from the reports of elevated serum antibodies to a variety of common viruses such as herpes simplex virus (HSV), rubella and coronavirus (2, 11, 212, 239). Paramyxovirus-like inclusions in MS brains have also been demonstrated by electron microscopy (112). A viral etiology has clearly been established in a variety of other human demyelinating diseases such as subacute sclerosing panencephalitis (SSPE) linked to measles virus (99), progressive multifocal leukoencephalopathy linked to JC virus (70) and HTLV-1 associated mylopathy/tropical spastic paraparesis (HAM/TSP) (179).

Some of the viruses that have been implicated in the etiology of MS include: human herpes virus-6 (59, 89), varicella zoster virus (205), vaccinia virus (3), measles virus (72) (42), canine distemper virus (80), parainfluenza virus (207), rubella virus (103) and retroviruses (115, 183). There has been no success in linking MS to a single virus and there is some speculation that MS could be the result of multiple virus infections of the CNS.

MECHANISMS BY WHICH VIRUS DAMAGE COULD OCCUR IN MS

The viruses that have been implicated in the etiology of MS could enter the CNS via the systemic circulation or through nerves. Once in the brain, the virus may mediate damage by any one of the following mechanisms (232).

1. Direct damage of the cells such as oligodendrocytes by cytolysis.
2. Viruses may set up a chronic infection of cells or could lead to persistent infection of the cells, altering the metabolism of cells such as oligodendrocytes that are important in the myelination process.
3. Viruses might induce an immune response to a viral antigen and by the process of 'epitope-spreading' may ultimately mediate the damage of self-components in the CNS.
4. Viruses could set up an autoimmune reaction against cells in the CNS by a process known as 'molecular mimicry'.
5. Viruses may lead to apoptosis of infected cells, leading to packaging of virus components along with self-components in the surface blebs of the apoptotic cells. The presentation of these novel packages to macrophages could lead to breakdown in immune tolerance against self-antigens and result in autoimmune disease.

MS PATHOPHYSIOLOGY– THEORETICAL PATHOGENESIS

T cells reactive against major constituents of myelin sheath, such as myelin basic protein (MBP) and proteolipid protein (PLP), are detected in specimens of peripheral blood from normal individuals but this activation is regulated by immunoregulatory

mechanisms. These myelin reactive T cells may become activated in the peripheral system by certain factors such as microbes, which show similarity to these epitopes by a process called molecular mimicry. MBP shows extensive homology to several common pathogens such influenza virus (219), Epstein-Barr virus (26) and herpes virus (40).

Following peripheral activation, these activated T cells are capable of transmigrating across the BBB via interactions with several adhesion molecules such as integrins and members of the immunoglobulin supergene family like CD4. These activated T cells also express antigens such as very late antigen (VLA)-4, which helps in binding to the endothelium (83). In MS lesions, T cells in the perivascular cuffs express VLA-4 and blockage of VLA-4 in the autoimmune model of MS- Experimental Allergic Encephalitis (EAE) prevents development of the disease process (255). In MS lesions, the inflamed endothelium expresses MHC class II molecules and vascular adhesion molecules (VCAMs) (140).

In the CNS, the T cells activate local antigen presenting cells such as microglia cells and astrocytes, which present peptide and lead to stimulation of the T cell in a positive feedback loop. The locally activated T cells then secrete proinflammatory and cytotoxic factors of the Th1 subtype mostly which recruit macrophages to the site of damage. In addition, a critical antibody response is directed against myelin and the complement cascade is also activated with membrane attack complexes directed against myelin. This orchestrated attack against myelin and the myelin forming cells by macrophages and their products such as TNF-□; B cells and antibodies against myelin and complement activated complexes, results in damage to myelin surrounding nerves

and results in areas of demyelination impairing saltatory conduction along the axon and resulting in the neurological deficits associated with the disease.

ANIMAL MODEL SYSTEMS OF MS

Models for MS fall into two categories: viral and non-viral. The viral model is based on the hypothesis that the etiological agent involved in triggering MS is a virus. It has the advantage that epidemiological and serum antibody evidence indicates viral infection as a trigger for this disease (229). But the main drawback is that no single virus has been isolated that could be implicated in the etiology of MS. However, several virus-induced models in experimental animals have been used to study this disease and address various questions relating to age, sex, and genetic basis of this disease. Some of the commonly used viral models for MS include:

1. Theiler's virus
2. Mouse Hepatitis virus
3. Semliki Forest virus
4. Visna
5. Canine Distemper virus

Of the non-viral models for MS, experimental allergic encephalitis (EAE) is the most studied model. In EAE, disease is initiated by extra neural injection of CNS material or purified CNS antigens emulsified in an appropriate adjuvant. This model enables investigators to ask very specific questions regarding the autoimmune component of the disease. The other less commonly studied models include injection of

various chemicals including ethidium bromide, cuprizone and lysolecithin. These have been particularly useful in the study of remyelination process in MS.

THEILER'S VIRUS

Theiler's murine encephalomyelitis virus (TMEV) is a member of the genus Cardiovirus in the family *Picornaviridae.* It is a single-stranded, non-enveloped, RNA virus. The genome of TMEV has been sequenced and infectious clones have been generated (30, 175). Serological evidence indicates that Mus musculus (house mouse) is the natural host for the virus, but it is also present in other species of mice, voles and rats. TMEV was first isolated by Max Theiler in 1930 from the CNS of mice with spontaneous flaccid paralysis of the hind leg (236). In 1952, Daniels et al. reported demyelination during TMEV infection (47). But the model did not gain much importance until Lipton, in 1975, reported a biphasic disease leading to demyelination (134). It has remained a very popular model for MS since then. Modes of infection of TMEV in its host include intra-cranial, intra-peritoneal and intra-nasal. TMEV is a natural enteric pathogen of mice and the incidence of spontaneous paralysis is low, about 1 in 2000. The rare natural incidence of CNS infection is similar to that seen in MS. It remains one of the most popular models for MS for the following reasons:

1. Chronic pathological involvement limited to the CNS white matter.
2. Myelin breakdown is accompanied by mononuclear cell inflammation.
3. Demyelination results in clinical disease for example spasticity by involvement of upper motor neuron systems.
4. Myelin breakdown is immune mediated.

5. Disease is under multigenic control with a strong linkage to certain MHC genes.
6. Axonal transection is also observed similar to MS.

TMEV can be divided into two subgroups on the basis of their neurovirulence properties (Table 1). The highly neurovirulent strains that comprise of at least 4 isolates: GDVII, FA, VIE 415_{HTR}, and Ask-1 viruses. GDVII and FA are the most commonly studied neurovirulent strains.

2. The less virulent TO strains, represented by DA, BeAn, Yale and WW.

Infection with the neurovirulent strains kills all strains of mice of all ages in a few days by a fatal polioencephalitis. The affected areas include hippocampus, cerebral cortex, basal ganglia, thalamus, brain stem and spinal cord (30, 137). The mice develop a hunched posture and limb paralysis and rapidly succumb to widespread lytic infection of neurons. Infection with the TO strains causes a biphasic disease that produces a chronic, immune-mediated, inflammatory demyelinating disease in susceptible strains of mice, showing similarity with MS. SJL/J and CBA mice are susceptible to TMEV infection and BALB/c and C57BL/6 are resistant (Table 2). The biphasic disease produced by the TO strain is well documented. In the natural course of infection, TMEV replicates in the gastrointestinal tract of the host and occasionally gains access to the CNS where it persists for the lifetime of the host. The route of entry of the virus into the CNS has not been clearly understood, although it is thought to spread to the CNS via retrograde axonal transport (146, 199), by transport within infected macrophages (37, 129) or by replication within the blood brain barrier (BBB) (119, 250, 251, 259).

TABLE 1. Characteristics of TMEV subgroups

	Neurovirulent subgroup	Avirulent subgroup
Representative strains	GDVII, FA	BeAn, DA, WW, Yale
Plaque size in BHK-21 cells	Large	Small
Disease phenotype caused	Acute polioencephalitis	Biphasic: early-acute polioencephalitis, late-chronic demyelination
Viral persistence	No	Yes
Cell tropism	Neurons	Neurons, glial cells

TABLE 2. Strain differences in susceptibility to TMEV-induced demyelination

Highly susceptibility	Intermediate susceptibility	Highly resistant
SJL	C3H	BALB/C
DBA/1	CBA	C57BL/6
DBA/2	AKR	C57BL/10
SWR	A	C57L
PL	C57BR	129/J
P		
NZW		

In the acute stage of the disease following intra-cranial infection with the TO strains, there is widespread infection of neurons. This occurs within the 10-12 days following intra-cranial infection and is characterized by a mild encephalomyelitis (134). Viral antigens and RNA have been demonstrated in neurons of the gray matter (GM) (46). There is some infection and destruction of astrocytes involved also, but oligodendrocytes are not involved in the acute stage of the disease (37, 46). The inflammation at this stage is largely restricted to the GM. In the resistant mice, like BALB/c mice, a strong inflammatory response is recruited to the CNS and the virus is totally cleared in 3-4 weeks. In the susceptible strains of mice like the SJL/J mice, the virus titer is reduced greatly, but some still persist reportedly in oligodendrocytes (166, 174), microglia or macrophages (37). This persistence gives rise to inflammatory lesions in the white matter (WM), mostly in the spinal cord, but also some in the brain. This summarizes the chronic stage of the disease in the susceptible mice that develops 1 month after infection and persists for the lifetime of the host. The distinctive change in the distribution of the virus as well as inflammatory response from GM to WM has been well appreciated but not clearly understood. During this stage, the mice develop a wobbling gait that develops into weakness in the posterior limbs followed by spastic paralysis. In the later stages, there is urinary incontinence and loss of righting reflex (135). In the chronic stage, the virus is methods such as reverse transcriptase polymerase chain reaction (RT-PCR) and plaque assay. The number of infected cells is low but they are usually present in small foci predominantly in the anterior and lateral columns of the thoracic part of the cord. There is extensive inflammation and loss of myelin during this

stage. This stage is characterized by meningeal infiltration; cuffs of mononuclear cells around blood vessels; and parenchymal infiltration consisting of large numbers of activated macrophages, T cells (both CD4+ and CD8+) and B cells. The demyelination corresponds strongly with the presence of inflammatory response. There is extensive loss of myelin around normal appearing axons. Damage to myelin has been contributed to several factors, which may act singly or in combination with other factors. Demyelination may result from direct viral infection and destruction of oligo-dendrocytes. TMEV causes lytic infection of oligodendrocytes in culture and during the chronic stage of infection with TO strains, viral antigens and apoptosis have been demonstrated in oligodendrocytes (241). Unlike the acute stage of infection, when virally infected neurons undergo apoptosis, there is no correlation between the apoptosis and viral infection in oligodendrocytes. Viral antigens and RNA have been demonstrated in oligodendrocytes suggesting a role for viral persistence in the CNS. In addition, the immune response may contribute to the demyelination process in numerous ways. Immunosuppression at this stage reduces the incidence of the disease. Time of administration is crucial as early immunosuppressive therapy might actually be more harmful as it will allow for excessive replication of the virus(252).

IMMUNE RESPONSE

In response to TO virus infection, virus specific humoral immune response starts 1week post infection and reaches a peak by 2 weeks and may be sustained for the lifetime of the host (184). Neutralizing and other virus specific antibodies have been detected. B cells and CD8+ cells play an important role initially in viral clearance. Both

susceptible and resistant strains of mice can be infected with the virus, but only those that can mount a strong anti-TMEV delayed type hypersensitivity (DTH) response develop inflammation and demyelination in the spinal cord WM (36). Most of the studies point to a strong role for Th1 type of immune response predominantly in the development of the chronic stage of the disease. IgG2a subclass of IgG is found predominantly in susceptible strains of mice with little IgM antibody detected by day 14-post infection (32, 184). IgG2a production may be an *in vitro* measure of the preferential stimulation of a Th1 pattern. CD4+ T cells of the Th1 subset mediate delayed type hypersensitivity (DTH) and regulates IgG2a production through IFN-γ. T cell proliferation and DTH appear by 2 weeks post infection and remain elevated for 6 months (36). Both are specific for TMEV and mediated by CD4+ MHC class II restricted T cells (36). There is also definite correlation between onset of demyelination and development of virus specific T cell response and DTH. DTH and T cell proliferation responses in SJL/J mice have been found to be primarily towards VP2 and specific to VP2 amino acids 70-85. VP2 70-86 is the immunodominant epitope in SJL/J mice responsible for immunopathology (61, 62). Mice mount cell-mediated immunity and humoral immunity early and decrease the viral titer 1000-10,000 fold but this still leads to persistence. Extraneural persistence, however, is not observed.

Susceptibility to viral persistence and chronic disease is genetic and it has been linked to a number of loci including major histocompatibility complex (MHC) H2D loci (36, 197); T cell receptor β-chain (155); Interferon-gamma (IFN-γ) (23, 29); carbonic anhydrase (156) and myelin basic protein (MBP) genes (23). Other factors such as sex,

age, stress and/or other infections in addition to dosage and strain of the virus may be predisposing factors (107, 199, 231). The brain-derived TO are more virulent than the tissue-adapted virus. TMEV are capable of infecting many cell types of numerous species including insect cell lines, but are capable of inducing demyelination only in mice.

DETERMINANTS OF NEUROVIRULENCE AND PERSISTENCE

A considerable amount of work has gone into understanding the differences between the persistent and the neurovirulent strains of Theiler's virus in order to address their differences in pathogenesis. TO strains lead to the persistence of the virus in various cell types. In the acute stage of the disease process, viral antigens have been demonstrated in neurons and astrocytes (24, 198, 241). During the early stages of the chronic disease, viral antigens have been demonstrated in macrophages, astrocytes and oligodendrocytes. In the later stages of the chronic phase, the distribution of virus is confined mainly to oligodendrocytes (241). GDVII causes fatal encephalitis and the virus is seen to cause lytic infection only in neurons. In the rare survivors of this encephalitic process, the virus does not persist in any cell type. The complete nucleotide sequences of representative strains for both TMEV subtypes are known and infectious cDNA clones of both viruses are available. BeAn and GDVII are 90.4% identical at the nucleotide level and 95.7% identical at the amino acid level (175, 188, 189). The changes are dispersed all along the genome. The most differences occur at the 5' end. The 3' end is more or less conserved between all groups within *Picornaviridae*. The 5' non-coding region of picornaviruses may have a number of functions relating to

secondary structure, which may in turn control viral RNA synthesis, initiation of translation, encapsidation, and virion uncoating. Hence this region may be very important in pathogenesis. Based on numerous investigations, a number of genomic regions and gene products have been postulated to influence virulence. Studies by Jnaoui and Michiels (97) suggest that GDVII and BeAn utilize partly distinct pathways for both entry into cells and genome replication and have suggested a major role for receptors in the pathogenesis of the two viruses. The role for receptors in the pathogenic process of the two viruses should not to be discounted. Several analyses of recombinant TMEVs have mapped persistence determinants to the capsid suggesting a role for a virus-receptor interaction in persistent infections. Several authors have shown that the two viruses utilize different mechanisms to enter a single cell line (58, 97). Low virulence BeAn and DA have been shown to use cell surface sialic acid as an attachment factor (257), while GDVII does not require sialic acid. Sialic acid plays an important role not just in cell entry but also in TMEV persistence and demyelinating disease (257). Outside the CNS, peripheral nerve protein PO has been suggested as a TMEV entry receptor (131). Very recently, Reddi and Lipton (192) reported that the highly neurovirulent GDVII utilizes heparan sulfate as an attachment factor. Both viruses have been postulated to utilize UDP-galactose transporter for cell entry and infection (78). This might be of importance as UGT locus lies very close to the clinical disease marker D11Mit179 on chromosome 11(12). The broad tropism of DA suggests either the use of several receptors or one receptor that is highly homologous across species.

Recombinant viruses constructed by exchanging corresponding genomic regions between the highly virulent GDVII and the less virulent BeAn or DA viruses has shed light on the role of these genomic regions and the role of the capsid proteins. But Lipton reports that attenuation of neurovirulence of GDVII was not sufficient to produce a persistent infection (30, 136). These results were confirmed by Jarousse et al. (91) where the neurovirulence of GDVII was attenuated by insertion in the 5' region. This still did not yield a persistent strain of virus.

Some of the earliest work regarding capsid proteins and their role in virulence or persistence had been done by McAllister et al. (151) and it was suggested that persistence and demyelination map to the regions coding for VP1 capsid protein. Recent work by Adami et al. (1) confirms these findings and the regions involved in persistence have been further mapped to VP2 puff and the VP1 loop regions. These results were confirmed by Wada et al. (247) by exchanging the Loop II of VP1 of DA strain with the Loop II of GDVII and this altered persistence and demyelination. VP1 and VP2 had earlier been reported to play an important role in viral persistence (257).

The evidence for a role of an out of frame protein – L* protein in persistence of the TO strains has been highly compelling. L* is a protein produced in only the TO strains as a result of an alternative, out of frame initiation site. In GDVII, the AUG is changed to ACG, which can very rarely serve as an initiation codon too. Several studies did with mutations in this gene yield conflicting results. DAL*-1 with a mutation introduced in the L* region, is no longer able to persist and this suggests a strong role for this protein in viral persistence (63). But the introduction of L* into GDVII does not

convert it to a persistent strain, but only enhanced the infection of macrophages by GDVII (245). Recent studies by Obuchi et al. (169) show the association of the protein with microtubules in infected cells suggesting its further role in pathogenesis and persistence. L* has also been postulated to play an important role by inhibition of the antivirus cytolytic T cell activity that normally clears the virus in the acute stage of the infection (133). L* facilitates the growth of the virus in monocyte or macrophage cell lines (170, 245). In addition, L* has anti-apoptotic activity (63).

Interestingly, reports by Michiels et al. (158) and others suggest that GDVII and BeAn did not evolve as two separate groups, but that GDVII potentially evolved from TO strains by losing some of their persistence characteristics to become a more virulent strain. There has been speculation that a difference in expression of viral polymerases might account for viral persistence. GDVII was reported to express more viral polymerases in the cells that it infects in the CNS, while during the chronic phase infection with BeAn, there was very little viral polymerase expression in the infected cells, though viral proteins could be demonstrated in them (104).

BLOOD BRAIN BARRIER

In the natural infection, TMEV replicates in the gastrointestinal tract of the host and occasionally gains access to the CNS where it persists for the lifetime of the host. The route of entry of the virus into the CNS has not be clearly understood, although it is thought to spread to the CNS via retrograde axonal transport, by transport within infected macrophages or by replication within the blood brain barrier (BBB). The BBB is composed of specialized cerebrovascular endothelial cells (CVE). On the abluminal

side of the CVE are astrocytes with associated pericytes, perivascular macrophages and fibroblasts. The BBB plays an important role in maintaining the immunologically privileged nature of the CNS, although alterations in the BBB allow the entrance of immune cells into the CNS. In CNS inflammatory diseases, like MS and TVID, the BBB is disrupted during an early stage of the disease process, and immune cells, particularly the T cells traverse the BBB, enter the CNS and mediate demyelination. The disruption of the BBB seems to be a crucial event in the development of the disease. Viral antigens have been detected early in the TVID in CVE and this may play a role in the development of the disease (259). In addition a number of viruses have been shown to penetrate the BBB by replication within the CVE (99, 102). Interestingly many of the viruses implicated in the etiology of MS or shown to cause demyelination in animals also infect CVE (100, 101).

The BBB is formed by specialized CVE that line the lumen of the blood vessels. The endothelial cells have relatively few pinocytic vesicles and a high resistance (1900units/cm2), features that contribute to the limited permeability of the BBB. In addition, they have very little MHC expression unlike endothelial cells that supply other organs. In most inflammatory conditions of the CNS like MS and TMEV, lymphocyte cuffing is observed around post capillary venules and this suggests that the main route of cellular migration (and viral migration in case of TVID) may occur across the BBB.

Heterotrophic brain transplants from susceptible (SJL/J) to resistant mouse strains suggest that regulation of susceptibility to disease resides at the level of cerebral endothelium and not within the CNS parenchyma or lymphoid/bone marrow-derived

compartment. Therefore, the interactions that occur at the BBB are critical in the development of inflammatory CNS disease.

A role for BBB in the disease process of MS is suggested by the fact that BBB dysfunction is associated with relapses in MS. In addition, BBB dysfunction has been reported to be an initial event in the development of MS lesions. Elevation of soluble adhesion molecules (218) and the role of nitric oxide (66, 214) in endothelial cells has been well documented in MS and strongly suggests a role for BBB in the disease process. Antibodies to CVE were detected in MS patients and rhesus monkeys injected with CVE were shown to develop EAE. In patients with MS, therapy with IFN-caused exacerbations, which may have resulted from increased MHC class II expression on CVE. And subsequently, antigen presentation, which would allow increased access of T cells into the CNS. Interestingly, cytotoxic activity against CVE has also been described in MS patients (235, 240).

A number of viruses that induce demyelination have been shown to infect the CVE. Vascular endothelial cell damage has been documented in hemorrhagic fever viruses (13), human immunodeficiency virus (HIV) (33) and measles virus (41). Therefore, the BBB may play a very important role in the viral infection of the CNS. CVE possess many specialized receptors that may function as viral receptors (68). Infection of the CVE may allow for the virus to gain access to the CNS or may result in up-regulation of expression of various molecules involved in lymphocyte adhesion (228). Several viruses are also able to replicate in the CVE and destroy them thus causing a breach in the BBB allowing the entry of more virus and inflammatory cells

into the CNS (13, 144). During virus infection of CVE, damage to the BBB may be due to virus replication, virus induced cytopathic effects or anti-endothelial cells antibodies. Very recently HIV (220), Influenza virus (85) and SIV (4) have been demonstrated to induce apoptosis in CVE thus causing damage in the BBB. In TVID, CNS endothelial cells have been demonstrated to contain viral nucleic acid. Also, enhanced MHC class II expression has been found during TMEV infection suggesting a role for CVE in TMEV infection (250, 251). There is strong evidence to suggest that TMEV may enter the CNS by infection of the CVE. CVE are permissive to TMEV replication both *in vitro* and *in vivo*.

VIRAL INFECTIONS OF THE CNS

Neurotropic viruses follow two basic pathways to gain access to the CNS: (i) neuronal pathway and (ii) hematogenous pathway. Rabies virus (124) and Herpes simplex virus (HSV) (145) are the prototype of virus infection of the CNS through peripheral neuronal spread. A few other viruses such as poliovirus (195) and reovirus (57, 162) that had earlier been thought to spread through the hematogenous route have now been shown to spread via peripheral neurons in experimental animals. The main nerves involved in this spread are peripheral and cranial nerves (163). Rabies spreads via myoneural infection. In the case of HSV, cranial nerves such as olfactory and trigeminal nerves play a crucial role in the transport of the virus to the CNS (163). The olfactory nerve is particularly unique in it that the olfactory neurons are not protected by the BBB and therefore provide a direct neuronal access to the brain.

In the case of the hematogenous spread, enterovirus and arboviruses are the prototype of viremic spread to the CNS. These viruses have to overcome several barriers before gaining access to the CNS. The first and the most important criteria is that the virus must bypass or attach to and enter a host epithelial cell. This cell must be permissive to the virus. The first signs of infection involve replication at the site of entry and a transient viremia. Infection of secondary tissue usually ensues. Most frequently, there is a spread to the lymph nodes or they can bypass this and enter the circulatory system and seed other tissues. Arboviruses, enteroviruses, measles virus and varicella virus follow this mode of spread and seed in a variety of other organs such as liver and spleen (102). A local immune response at this point can curtail the spread of most viruses. Some viruses can, however, resist phagolysosomal degradation and then use the macrophages to circulate and replicate within.

Some viruses such as human rhinovirus, influenza virus and poliovirus have developed unique abilities to escape the immune system. These viruses have their receptors embedded in pits or 'canyons' in the viral membrane thus enabling them to evade the immune response (82, 127).

The primary viremia seeds the virus to distant locations. In neonatal HSV, virus infection of the CNS follows primary viremia. Virus must localize in the vessels of the CNS before crossing the BBB or the blood-CSF barrier. The virus can enter the CNS by either one of the following ways; by infection and replication within the CVE, by leaking across a damaged CVE, by passive channeling through the CVE (pinocytosis or colloidal transport) or by bridging endothelial cells within migrating leucocytes (233,

253). Either cell-associated or cell-free virus can cross the endothelial cells and enter the parenchyma or CSF.

APOPTOSIS

Apoptosis, or programmed cell death, has recently gained immense importance due to its role in neural development and in a wide variety of neurological diseases including viral infections. The term 'apoptosis' is derived from a Greek word and it implies leaves falling from a tree. It was coined in 1972 by Kerr, Currie and Wyllie to describe the common morphological changes that characterize the process of cellular self-destruction (110). It is a naturally occurring process and its importance has been recognized in embryogenesis, maintenance of homeostasis, normal cell turnover, induction and maintenance of immune tolerance and development of the nervous system.

Apoptosis is an active, energy-requiring process, which requires the synthesis of a series of proteins. In apoptosis, there is an orderly execution of death signals that ultimately results in the death of the cell, which is usually phagocytosed by a macrophage. This helps limiting the inflammatory response and results in very specific macrophage mediated removal of the apoptotic cell and remnants. Another form of cell death from which apoptosis needs to be differentiated is necrosis, which is caspase-independent, lacks DNA fragmentation and there is no formation of apoptotic bodies (Table 3). This results in leakage of intracellular contents to neighboring tissue and results in the elicitation of a huge inflammatory response Macrophages recognize apoptotic bodies that express phosphotidylserine and other surface molecules with the help of receptors such as CD14, scavenger receptors and integrins (157).

TABLE 3. Differential features and significance of necrosis and apoptosis

Apoptosis	Necrosis
Morphological Features	**Morphological Features**
Loss of membrane integrity	Membrane blebbing but no loss of integrity
Begins with swelling of cytoplasm and mitochondria	Begins with shrinkage of cytoplasm and condensation of nucleus
Ends with total cell lysis	Ends with fragmentation of cell into smaller bodies
Disintegration of organelles	Formation of membrane bound vesicles (apoptotic bodies)
	Mitochondria becomes lealy due to pore formation involving proteins of Bcl-2
Biochemical Features	**Biochemical Features**
Loss of regulation of ion homeostasis	Tightly regulated process involving activation and enzymatic steps
No energy requirement	Energy dependent active process
Random digestion of DNA (smear of DNA after agarose gel electrophoresis)	Non-random mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis)

TABLE 3. (contd)

Apoptosis	Necrosis
Post-lytic DNA fragmentation	Pre-lytic DNA fragmentation Release of various factors (cytochrome-C, AIF) into cytoplasm by mitochondria Activation of caspase cascade Alteration of membrane asummetry (translocation of phoshotidylserine from the cytoplasm to the extracellular side of the membrane)
Physiological Significance	**Physiological Significance**
Affects groups of contigious cells	Affects individual cells
Evoked by non-physiological disturbances (complement attack, lytic viruses, hypoxia, metabolic poisons)	Induced by physiological stimuli (lack of growth factors, changes in hormonal environment)
Phagocytosis by macrophages	Phagocytosis by macrophages of adjacent cells
Significant inflammatory response	No inflammatory response

Apoptosis can be initiated by various stimuli including viral infection. The apoptotic process can be divided into 2 phases: (i) initiation phase, which depends on apoptosis inducing stimuli (ii) effector phase, common to all specific process. Both these phases involve caspases and caspase-3 has a key role in the effector phase. Caspases are central to the apoptotic process in most cases, but there are some caspase independent proteases such as calcium activated cysteine protease- calpain and the ubiquitin dependent proteasome pathway that can play an important role too. DNA fragmentation, translocation of PS to the outer side of the cell membrane, morphological changes are all outcomes of the two phases. In most cases, the apoptotic cell death begins 12-24 hrs after the initiating trigger.

A cell undergoes apoptosis following some common conditions such as withdrawal of positive signals (growth factors for neurons, IL-2); receipt of negative signals (increased levels of oxidants within the cell, damage to the DNA by UV, X-ray or chemotherapeutic drugs) or by molecules that bind to receptors and signal death of the cell (TNF, lymphotoxin, FasL binding to Fas).

The first description of virus-induced apoptosis was the finding that the E1B-19K mutant of adenovirus induced severe cytopathic effect of the infected cells, accompanied by a marked decrease in the yield of the progeny virus (190). Similar results were observed in baculovirus infected insect cell lines and other mutant adenovirus strains (117). The effect of apoptosis is time dependent and it may act in favor of the host or in the favor of the virus. If the host cell manages to induce apoptosis before the virus has completed a round of replication, the cell may curtail spread of the virus. In this case,

some viruses have developed specialized ways of overcoming this by producing anti-apoptotic products. However, if there is induction of apoptosis after the virus has completed a few rounds of replication, this may be beneficial to the virus and aid in further spread of the virus throughout the system.

A number of DNA and RNA viruses, including varicella-zoster (208), human immunodeficiency virus (125), coronavirus (20) and reovirus (48), have been reported to trigger apoptotic cell death. In contrast, other viruses, such as adenovirus (190), baculovirus (39), vaccinia virus (60), and Epstein-Barr virus (71), are capable of down-regulating the apoptotic process to allow for cell survival and continued viral replication. Poliovirus is unique in its ability to induce apoptosis as well as inhibit the process (238). The relation between induction of apoptosis and neurovirulence is highly conflicting. Itoh et al. report that in case of Sendai virus infection, the virulent strain of the virus causes minimal apoptosis resulting in a high virus titer (86). Whereas, the avirulent strain induces apoptosis and this occurs before the virus completes a round of replication resulting in low virus titers. In the case of Sindbis virus, which causes fatal encephalitis in neonatal mice, neurovirulence has been associated with apoptosis (130, 243).

Interestingly, virus-induced apoptosis can occur by different mechanisms in insect and in mammalian cell lines. Apoptosis induced by HIV-1 infection of the CNS has been well documented. Recent reports have demonstrated apoptosis of neurons, astrocytes and endothelial cells in HIV-1 infection (220). Apoptosis of CVE has been suggested to be an important factor in the development of disease in HIV-1 and Influenza A virus infection of the CNS (85).

Virus-induced apoptosis in cells can have a variety of effects. The most common one being the induction of cytopathic effects (CPE) in cells. CPE by viruses results in disruption of a variety of cell processes such as nucleic acids and proteins, cytoskeletal architecture and the presence of membrane integrity. Viruses such as Herpes simplex virus, HIV, Influenza and Measles virus are capable of inducing CPE by apoptosis (182). Rosen et al. also suggest that infection with Sindbis virus can lead to an autoimmune process through the induction of apoptosis (204). In their classic set of experiments they indicate that there was distribution of viral antigens in surface blebs of apoptotic cells. They proposed that this could define a novel immune context for self-antigens. In the case of Systemic Lupus Erythematosus (SLE), calreticulin, an endoplasmic reticulum auto antigen has been shown to form complexes with rubella during virus replication. These studies strongly suggest that induction of apoptosis could generate novel sources of concentrated foreign and self-antigens, which could break down tolerance and lead to autoimmunity.

Recent evidence indicates that apoptosis or programmed cell death plays a very important role in the development of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (96, 171, 178). The role of apoptosis in the disease process of MS is currently being investigated and there are reports of oligodendroctye and astrocyte apoptosis in MS (176, 200, 258). Apoptosis of oligodendrocytes (241) and neurons (10, 241) has been documented in TVID. Whether this plays an important role in the disease progression has yet to be determined.

TMEV AND APOPTOSIS

TMEV from both neurovirulence groups have been shown to induce apoptosis both *in vitro* and *in vivo*. Whether this is important in the pathogenesis of the virus has yet to be determined. Tsunoda et al., showed that in the acute disease by both GDVII and DA, there is induction of apoptosis of neurons (241). The induction of apoptosis by GDVII could possibly explain the development of fatal encephalitis by this strain. At this stage, viral antigens co-localize with neurons undergoing apoptosis (241). It is postulated that reduced apoptosis or survival of neurons in DA may be due to apoptosis inhibiting molecules. Ghadge et al. showed the role of L* - an out of frame protein, in the persistence of DA (63). GDVII lacks L* and is more efficient at inducing apoptosis on permissive BHK21 and restrictive macrophages. L* appears to play a very crucial role in persistence as DA L*, a mutant that lacks L* now shows characteristics very similar to GDVII (245). L* has anti apoptotic properties adding credence to the fact that persistence might be linked to a greater ability to inhibit apoptosis by the persistent strains of TMEV (63).

Extensive experimentation indicates that GDVII is more capable than the TO strains in inducing apoptosis (95, 173, 241). Both restrictive as well as permissive cells undergo apoptosis on infection with GDVII and BeAn *in vitro* (10, 92-95). During the acute phase of the disease by GDVII and TO, apoptotic neurons are detected and there is co-localization of the viral antigen and apoptotic cells indicating, induction of apoptosis to be a more direct effect of the virus and less of external factors (241). But during the chronic stage in TO infection, the scene appears to be different. The sites of viral

persistence during the late demyelinating phase have yet to be resolved. Astrocytes, oligodendrocytes and macrophages all carry a major viral load and undergo apoptosis. There appears to be a lack of co-localization of viral antigen in these cells and induction of apoptosis indicating the involvement of soluble factors in apoptosis. Apoptosis induced in the neurons during the acute phase might follow a different pattern than during the chronic stage of TO infection. In oligodendrocytes, it is postulated that nitric oxide (87, 177, 203), cytokines and chemokines might play a role (111). Reduction of demyelination in SJL/J mice treated with TGFB2 shows the role of soluble mediators in TMEV infection (50). TGFB2 reduces demyelination by reducing macrophage dependent apoptosis though TGFB2 did not directly reduce TMEV. A role for nitric oxide in TMEV infection further implies the role for soluble factors in the induction of apoptosis. Increased level of nitric oxide leads to necrosis and low levels of nitric oxide leads to apoptosis in neurons. Inhibition of nitric oxide by amino guanidine (AG) reduced apoptosis but AG did not have anti-viral effect (203). This indicates the role of soluble factors and other elements of the immune system in the chronic stage of the disease.

BeAn and GDVII can both induce apoptosis in restrictive cells and permissive cells. But GDVII is 50 times more capable of inducing apoptosis in restrictive cells. It was earlier believed that TMEV was capable of inducing apoptosis only in restrictive cell lines and not in permissive cell lines, but studies on cerebellar explant culture of neurons have demonstrated that permissive cells also undergo apoptosis (10). In some cells, such as macrophage cell lines, the stage of differentiation determines whether the

cell can undergo apoptosis induced by TMEV (92). Myeloid precursor cell line M1 is protected from apoptosis by TMEV, whereas the differentiated M1D cells undergo apoptosis. Whether the state of differentiation of cells is important in the induction of apoptosis needs further verification in other cell lines. Difference in the induction of apoptosis in M1 and M1D cells has been linked to increased Bcl-2 expression in M1 cells than M1D. Also, M1D shows increased expression of pro-apoptotic proteins bax and bak.

The pathway of induction of apoptosis in cells infected by TMEV has only been partly identified. The pathway may be different in various cell types. However, there is compelling evidence that apoptosis is an intrinsic mechanism. UV-inactivation of TMEV leads to decreased apoptosis in various cell types (95). Recent studies have indicated that in a macrophage cell line, BeAn induces caspase-3 dependent apoptosis (94). The other proteins involved are TRAIL and TNF-□. The same studies have also shown that over-expression of Bcl-2 had no effect on cell viability or caspase-3 production.

HYPOTHESIS

The hypothesis tested in this dissertation is that TMEV is capable of inducing apoptosis in CVE and this may play a crucial role in the disruption of the BBB. GDVII is more effective in inducing apoptosis in the CVE and hence may be responsible for the fatal encephalitis in all strains of mice. BeAn, in contrast, shows reduced ability to induce apoptosis in CVE as compared to GDVII and this may account for the ability of BeAn strain to establish a persistent infection in cells. The differential induction of apoptosis by the two strains may account for the difference in their neurovirulence properties.

Experimentally addressing the mechanisms involved in the induction of apoptosis in CVE by TMEV may provide insights into the neuropathogenesis of Theiler's virus. Induction of apoptosis in CVE by TMEV was assessed by a variety of methods.

Specific questions addressed were:

- Differences between induction of apoptosis in CVE by neurovirulent and neuropersistent strains of TMEV
- Correlation between induction of apoptosis and cytopathic effects of the virus
- Dependence of induction of apoptosis on the MOI of the virus
- Pathway of apoptosis involved in the induction of apoptosis
- Correlation between *in vitro* and *in vivo* findings

CHAPTER II

INDUCTION OF APOPTOSIS IN CLONED MOUSE CEREBROVASCULAR ENDOTHELIAL CELLS FOLLOWING TMEV INFECTION

INTRODUCTION

Multiple Sclerosis (MS) is one of the most common demyelinating diseases affecting 250,000 individuals in the US alone (88). The etiology of MS is largely unknown, but epidemiological evidence suggests it to be an acquired environmental, possibly, viral disease (6). Several viruses have been implicated in the etiology of MS. One of the prominent features of MS is the breakdown of the blood brain barrier (BBB) early in the disease process (109). The BBB is responsible for maintaining the immunologically privileged nature of the central nervous system (CNS)(18, 90) and is composed of specialized cerebrovascular endothelial cells (CVE). Many viruses have been shown to enter the CNS following infection of the CVE (Johnson 1982). Interestingly, many of the viruses implicated in the etiology of MS or shown to cause demyelination in animals also infect CVE (6, 105, 113).

Theiler's-virus induced demyelination (TVID) serves as a relevant model for MS. Theiler's murine encephalomyelitis virus (TMEV) is an RNA virus belonging to the genus Cardiovirus in the *Picornaviridae* family. They are divided into 2 subgroups on the basis of their neurovirulence properties; (i) neurovirulent strains, represented by

GDVII and FA, cause a fatal encephalitis in all strains of mice and (ii) persistent TO strains, represented by BeAn and DA, cause a biphasic disease leading to demyelination in susceptible strains of mice. TMEV naturally infects the gastrointestinal tract of mice and occasionally gains access to the central nervous system (CNS) where it establishes a persistent infection.

The route of entry of TMEV into the CNS has not been determined. Some of the proposed portals of entry include (i) through infected macrophages (37, 129); (ii) retrograde axonal transport (146, 199) or (iii) by replication in the cells of the blood brain barrier (BBB)(119, 250, 251, 259). CNS endothelial cells have been demonstrated to contain viral nucleic acid in nude mice infected with TMEV (259). Also, enhanced MHC II expression has been found during TVID *in vivo* suggesting a role for CVE in TMEV infection (202). CVE are permissive to TMEV replication both *in vitro* (119, 251) and *in vivo* (259) and there is strong evidence to suggest that TMEV may enter the CNS by infection of the CVE. Infection of the CVE may allow the virus to gain access to the CNS and/or may result in the up regulation of expression of various molecules involved in lymphocyte adhesion.

In addition to infecting the CVE, several viruses are capable of inducing apoptosis. A number of DNA and RNA viruses have been reported to trigger apoptotic cell death in a variety of cell types. Several members of the *Picornaviridae* family such as Coxsackie virus B3 (31), Hepatitis A virus (22), Avian encephalomyelitis virus (138), Enterovirus 71(118) and Polio virus (8, 44, 139) are also capable of inducing apoptosis. Polio virus is unique in its ability to both induce apoptosis as well as prevent apoptosis

(238). In the case of Sindbis virus, which causes fatal encephalitis in neonatal mice, increased neurovirulence has been associated with apoptosis (130). Recent reports have demonstrated apoptosis of the neurons, astrocytes and endothelial cells in Human Immunodeficiency virus (HIV-1) infection (220). Apoptosis of CVE has been suggested to be an important factor in the development of disease in HIV-1 (33, 220), Simian Immunodefeciency virus (SIV)(4) and Influenza A virus (85) infection of the CNS. TMEV from both neurovirulence groups have been shown to induce apoptosis both *in vitro* (10, 92-95) and *in vivo* (241). A number of cells including macrophages, astrocytes, neurons and oligodendroctyes have been documented to undergo apoptosis during TMEV infection (10, 92, 95, 180, 241).

The aim of the present study was to study the induction of apoptosis in cloned mouse CVE derived from two strains of mice (SJL/J – prototype strain susceptible to TVID and BALB/c – prototype strain resistant to TVID). Induction of apoptosis in the CVE was studied following infection with GDVII and BeAn strains of Theiler's virus by a variety of methods in order to determine whether neurovirulence correlated with increased levels of apoptosis.

MATERIALS AND METHODS

Virus

The BeAn 8386 strain and GDVII were obtained from Dr. Howard L. Lipton (Northwestern University, Evanston, IL). The virus was grown in BHK-21 cells, and the culture supernatant containing infectious virus was aliquoted and stored at -70C before use. The viral titer was determined by plaque assay on BHK-21 cells (206).

Cell culture

CVE that have been isolated from brain microvessels of either TVID-susceptible (SJL/J) or resistant (BALB/c) mice were used in these experiments. CVE were isolated and cloned by limiting dilution as described previously (213). These cells remain diploid and maintain their differentiation markers in culture. Frozen aliquots were thawed and grown in Iscove's Modified Dulbecco's Medium (IMDM)(GIBCO BRL, New York, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 2mM glutamine (GIBCO BRL, New York, NY), 100units/ml Penicillin and 100 g/ml Streptomycin (GIBCO BRL, New York, NY) at 37°C and 4%CO_2.

Infection of CVE

SJL/J and BALB/c CVE were grown in IMDM supplemented with 10% FBS for growth and 1% FBS for post inoculation maintenance of cell cultures. For infection with virus, the CVE were grown to confluence in T 25 flasks and infected with BeAn and GDVII strains of Theiler's virus at a MOI of 1. The viruses were allowed to adsorb for 45 min at room temperature and were subsequently washed with IMDM and further incubated in IMDM with 1% FBS. Control cells were treated similarly but without the addition of

the virus. The cells were resuspended using Trypsin and EDTA at the required time points and utilized for the respective assays.

UV-inactivation of the virus

Inactivation of both BeAn and GDVII was carried out by exposing previously titered virus infected BHK cell lysates to the UV illuminator lamp (1330 W/cc) at a distance of 13cm for 30 min. UV-inactivation of the virus was confirmed by performing plaque assay.

Annexin assay

The translocation of phosphotidylserine from the inner side of the plasma membrane to the outer layer is an early apoptotic plasma membrane alteration. After the respective treatment with either virus, the CVE were stained with the Annexin V-FLUOS staining kit (Boehringer Mannheim, Indianapolis, IN) and quantified by fluorescence activated cell sorter (FACS Calibur, Becton Dickinson, San Jose, CA). The staining was performed as per the manufacturer's recommendations. The proportion of cells stained by propidium iodide (indicating necrosis), Annexin V (indicating apoptosis), or both (late stages of apoptosis) was determined using an Epics Coulter cytometer (Becton Dickinson, San Jose, CA).

LSC^R analysis

Cells prepared by the method above were also analyzed by the Laser Scanning Cytometer (LSC^R system). 10 l of each sample at a concentration of $1x10^6$ cells/ml were utilized for LSC^R analysis. The LSC^R utilizes an argon laser operating at 5mW. The cell detection threshold was set to select single cells based on forward angle scatter

integral. Cells from the region determined were electronically gated to a dot plot of Annexin V integral versus PI integral. Cells were gated based on Annexin V staining and PI staining. Cells from each region were sorted and plotted by Compu Sort™.

Electron microscopy

SJL/J and BALB/c CVE were infected with BeAn and GDVII as previously described. At 18h and 24h post infection, the cells were harvested, pelleted by low speed centrifugation (1,000 rpm for 10 min) and fixed in 2% paraformaldehyde, 2% gluteraldehdye fixative in 0.2 M Cacodylate buffer. After primary fixation, cells were pelleted, suspended in 1% osmium tetroxide, dehydrated in a series of 75-100% ethanol and propylene oxide and embedded in epoxy resin. Ultra thin sections were prepared in a Sorvall MT 2B ultramicrotome, stained with lead citrate uranyl acetate, and examined using a Zeiss 10C electron microscope.

DNA laddering

At the appropriate time points post infection, the DNA was extracted from $1x10^6$ mock infected and virus infected CVE using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. The extracted DNA was precipitated with ethanol, dissolved in TE and 1 g of DNA was digested at 37°C for 15min with 1 U of DNase-free RNase (Promega, Madison, WI). DNA fragments were resolved by agarose gel electrophoresis and stained with Syber Gold (Molecular Probes, Eugene, OR) to visualize fragmented DNA.

DNA.Hoechst, mitochondrial activity and caspase-3 staining

Monolayers of CVE cells grown on glass coverslips were mock-infected or infected with BeAn or GDVII at MOI of 1. At different times p.i., cells were washed in PBS and stained with the appropriate dyes.

Hoechst staining

Hoechst 33342 is a vital DNA stain that binds preferentially to A-T base pairs. Cells undergoing apoptosis can be visualized showing cell shrinkage and chromatin condensation. The cells following infection were treated with 5μM of cell-permeable Hoechst 33342 (Molecular Probes, Eugene, OR) for 15 min in the dark, mounted on slides in IMDM and visualized under fluorescence microscope. For Hoechst 33342, the excitation was 340-380 nm and the emission filter was 465 nm. Multiple cells were inspected randomly, and only representative fields are presented in the figures.

Mitochondrial activity staining

The accumulation of tetramethylrhodamine methyl and ethyl esters in mitochondria and the endoplasmic reticulum has also been shown to be driven by their membrane potential. Tetramethylrhodamine, methyl ester, perchlorate (TMRM) has been used to measure mitochondrial depolarization related to cytosolic Ca2+ transients and to image time-dependent mitochondrial membrane potentials. At different times p.i., cells were washed in PBS and stained with 1.5 nM tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR) for 30 min at 37C and washed twice with phosphate buffered saline (PBS) (187). Images were acquired with a fluorescence microscope. For TMRM, the excitation was 568 nm and the emission filter was 590-640 nm. Multiple

cells were inspected randomly, and only representative fields are presented in the figures.

Caspase-3 staining

Rhodamine 110, bis-L-aspartic acid amide (R-22122) (Molecular Probes, Eugene, OR) contains rhodamine fluorophore flanked by aspartic acid residues and serves as a proteinase substrate for caspase-3. At different time points, the cells are stained with Rhodamine 110, bis-L-aspartic acid amide at 37C for 30 min in the dark. For Rhodamine 110, the excitation was 498 nm and the emission filter was 521 nm. Multiple cells were inspected randomly, and only representative fields are presented in the figures.

Caspase inhibitors

Inhibition of caspase activation was conducted using the pan caspase inhibitor-benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (ZVAD.FMK) (BIOMOL, Plymouth meeting, PA). zVAD.FMK at concentration of 100 M has been shown to inhibit caspase activity completely in cultured mammalian cells (226). Mock infected and BeAn infected cells at 90% confluency were treated with 100 M of zVAD.FMK for 1h prior to infection with the virus. Following viral infection, 100 M zVAD.FMK was added to the media. The cells were stained for Hoechst staining as described above following 24h of infection.

RESULTS

Serum deprivation induces apoptosis in CVE

The translocation of phosphotidyl-serine (PS) is one of the earliest markers for apoptosis and we recorded apoptosis using the staining of PS by Annexin V. Firstly, the induction of apoptosis in CVE due to serum deprivation was determined by growth of CVE in media containing decreasing amounts of FBS. Serum deprivation has been shown to be a strong signal for apoptosis in other cell cultures (52, 108). CVE were grown in media containing 0%, 1%, 5% and 10% of FBS and induction of apoptosis was recorded. There was some induction of apoptosis in CVE upon serum deprivation (Fig. 1). BALB/c CVE was more affected by serum deprivation than SJL/J CVE. CVE infected with either strain of Theiler's virus were incubated in media with 1% of FBS. The induction of apoptosis at this concentration of FBS was minimal and did not affect our readings.

Increasing MOI of the virus, increases CPE and apoptosis

Different authors use varying titers of virus for infection depending on the cell type. In order to determine the appropriate MOI for our studies on induction of apoptosis, SJL/J and BALB/c CVE were infected with BeAn and GDVII at increasing MOI (0.1, 1, and 10) for 24h p.i and the effects on apoptosis and CPE were recorded. Apoptosis was determined by Annexin V and CPE was recorded using a light microscope. Figures 2 and 3 indicate that increasing MOI shows increasing CPE and increased apoptosis. These results suggested the use of MOI of 1 for all our further studies. Virus at this MOI did not greatly induce apoptosis or cause increased CPE.

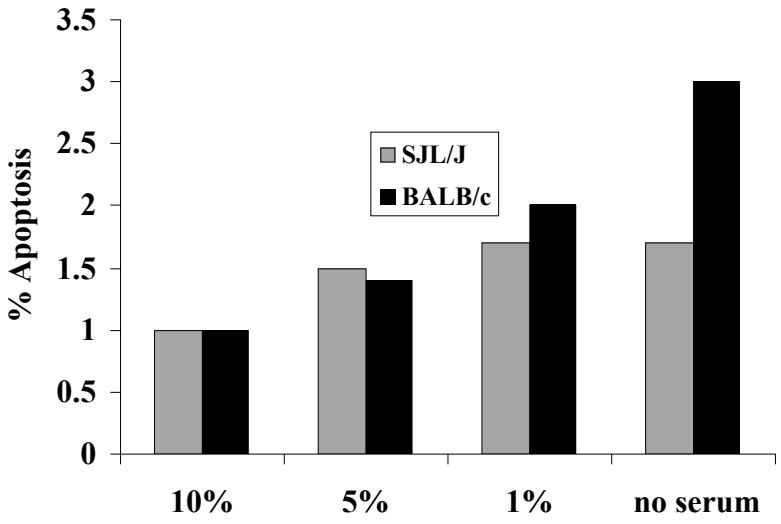

FIG. 1. Effect of serum deprivation on the induction of apoptosis in CVE. SJL/J and BALB/c CVE were treated with 10%, 5%, 1% and no serum for 24h and induction of apoptosis was analyzed by AnnexinV-PI staining. During viral infection, CVE were treated with 1% serum. This level of serum deprivation only caused minimal effects of apoptosis.

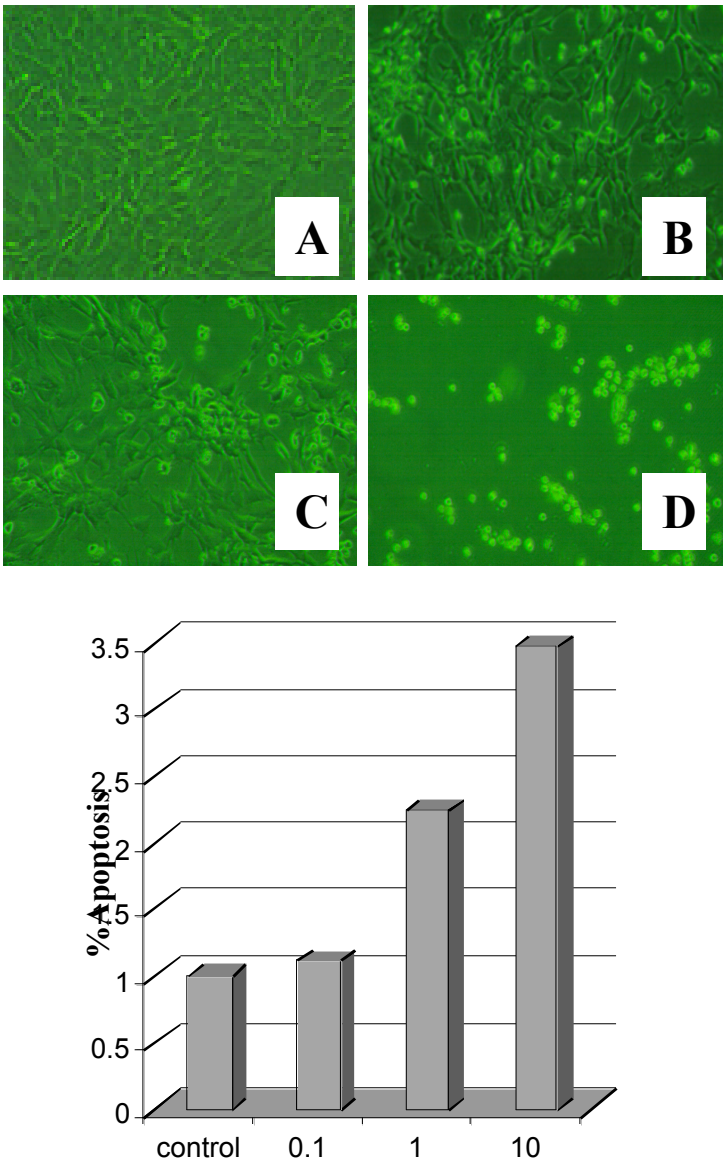

FIG. 2. Increasing MOI of BeAn increases CPE and apoptosis. Light microscopy (40X) of mock-infected SJL/J CVE (A) or SJL/J CVE infected with BeAn at MOI 0.1 (B), 1 (C) or 10 (D). (E) indicates Annexin V staining of CVE treated with increasing MOI of BeAn.

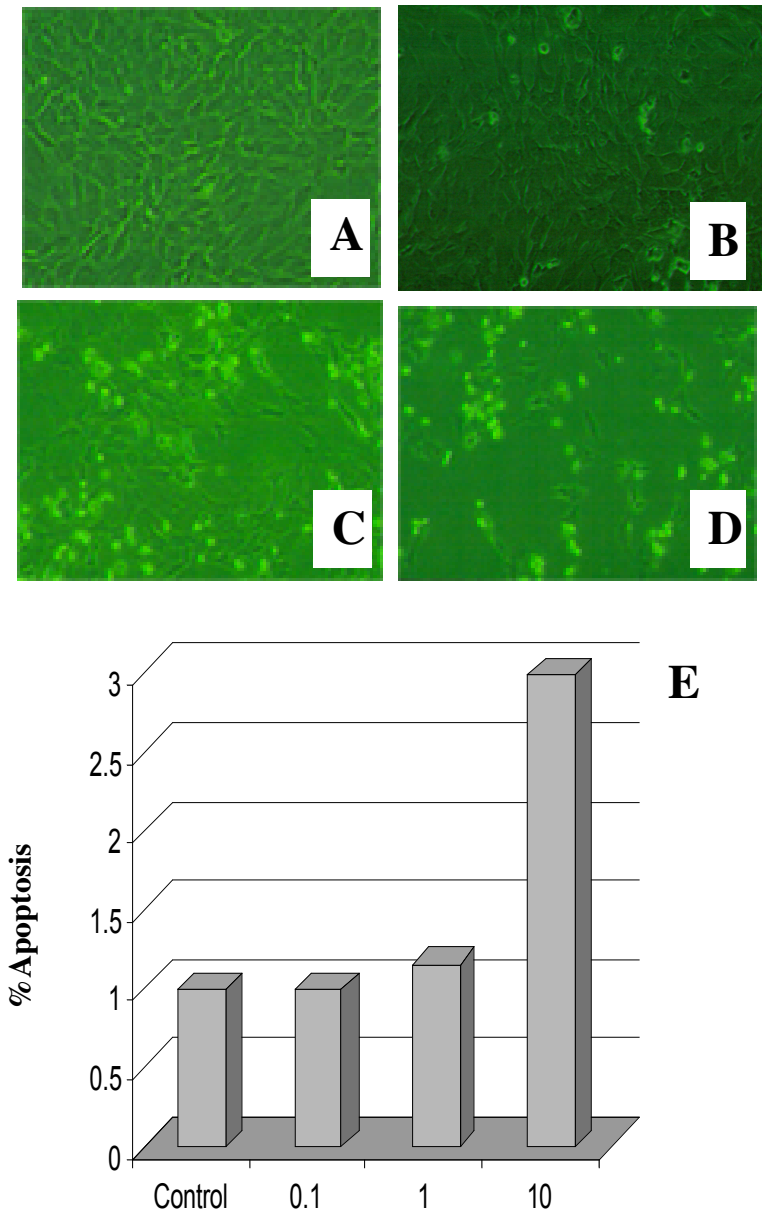

FIG. 3. Increasing MOI of GDVII increases CPE and apoptosis. Light microscopy (40X) of mock-infected SJL/J CVE (A) or SJL/J CVE infected with GDVII at MOI 0.1 (B), 1 (C) or 10 (D). (E) indicates Annexin V staining of CVE treated with increasing MOI of GDVII.

UV-inactivation of BeAn and GDVII reduces the ability of Theiler's virus to induce apoptosis

To determine whether the pro-apoptotic effect of BeAn and GDVII was receptor mediated or it required viral replication, we determined induction of apoptosis in SJL/J and BALB /c CVE following infection with UV-inactivated BeAn and GDVII. The UV-inactivation procedure was confirmed by plaque assay, which did not yield any plaques. Figure 4 indicates that UV-inactivation of BeAn and GDVII reduced the ability of the viruses to induce apoptosis as recorded by Annexin V assay.

Annexin V analysis of BeAn and GDVII induced apoptosis of SJL/J and BALB/c CVE

SJL/J and BALB/c CVE were infected with BeAn and GDVII at MOI of 1 and sampled at the following time points: 12h, 18h, 24h and 48 h. The induction of apoptosis was recorded by Annexin V assay. Figure 5 shows an increasing trend in the induction of apoptosis over time by infection of both CVE with BeAn and GDVII. There was minimal induction of apoptosis at 12h, but by 18h there was definite induction of apoptosis. It is interesting to note in figure 6 that at 24h, majority of the cells are in the apoptotic quadrant but by 48h, most of the cells have moved into the necrosis/late apoptosis quadrant indicating secondary necrosis. Figures 7 and 8 confirm these results. These findings were further confirmed by microscopic analysis of Annexin V stained cells using the Laser Scanning cytometer. Apoptotic cells stain green and necrotic cells stain red due to PI. Figure 9 shows cells early in apoptosis staining with Annexin alone and cells late in apoptosis stain with both Annexin and PI, indicating secondary necrosis.

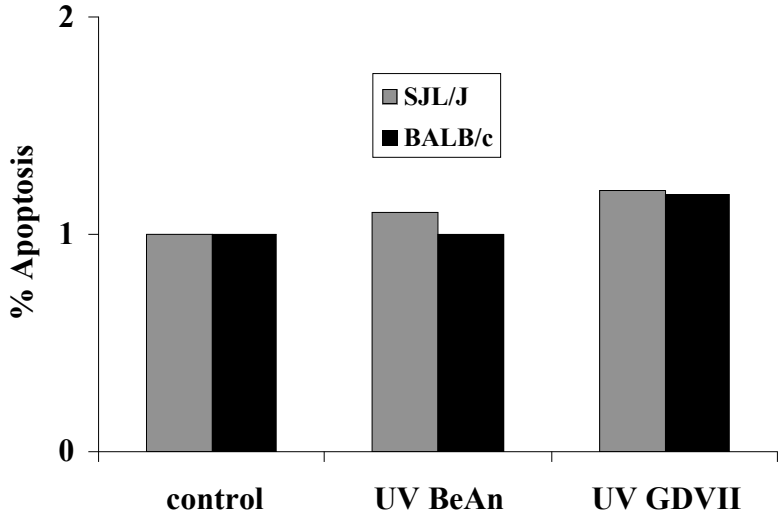

FIG. 4. Annexin V staining of CVE infected with UV-inactivated TMEV. SJL/J CVE and BALB/c CVE were infected with UV-inactivated BeAn and GDVII at MOI of 1 for 24h. Note that UV-inactivation of the virus reduces the ability of the virus to induce apoptosis. The above figure is representative of three independent experiments.

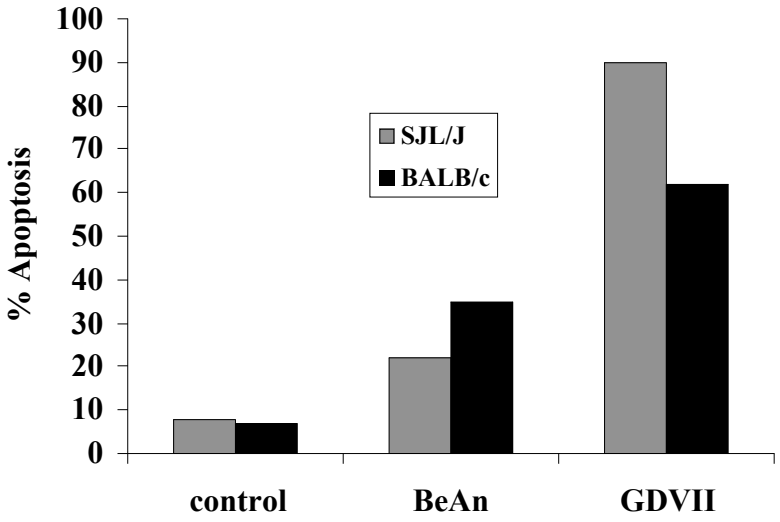

FIG. 5. Annexin V staining of TMEV-infected CVE (24h). SJL/J CVE and BALB/c CVE were infected with BeAn and GDVII at MOI of 1 for 24h. Note that both BeAn and GDVII were capable of inducing apoptosis but GDVII was a more potent inducer of apoptosis. The above figure is representative of three independent experiments.

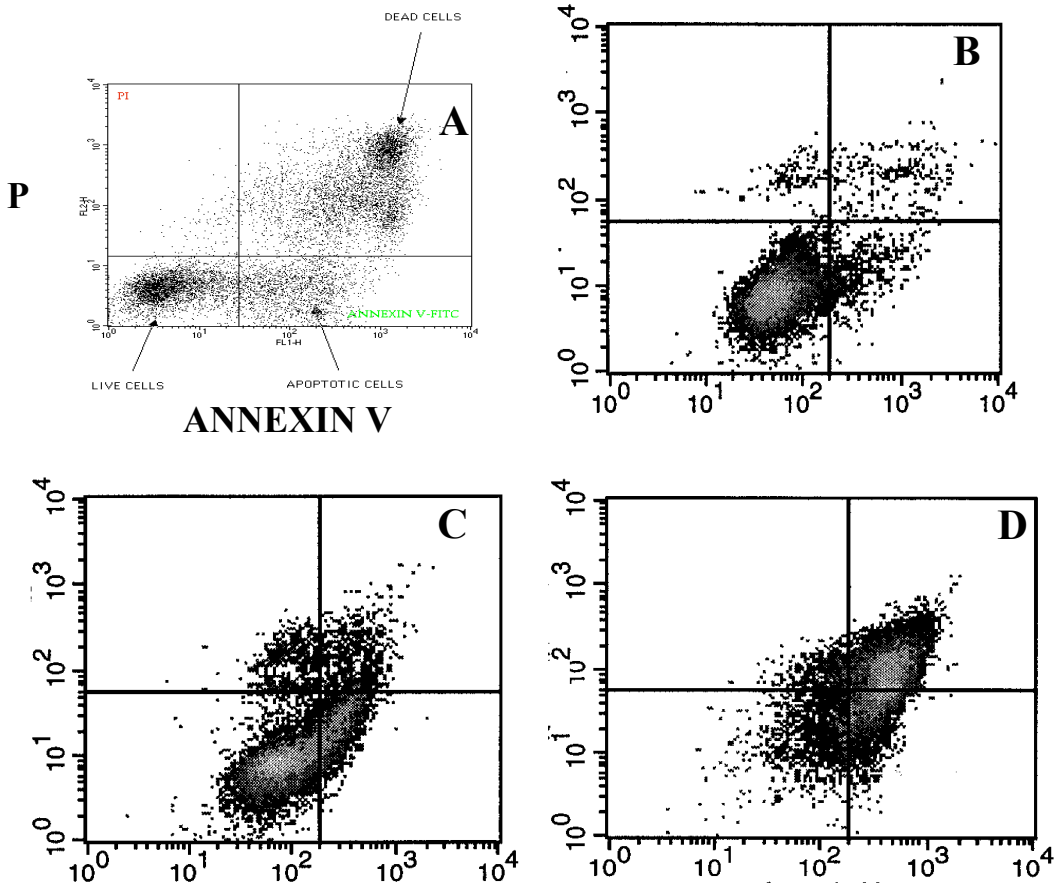

FIG. 6. Flow cytometry analysis of TMEV-infected SJL/J CVE (24h). SJL/J CVE were either mock-infected (B) or infected with BeAn (C) or GDVII (D) at MOI of 1 for 24 h and stained with Annexin V and PI as described in materials and methods. (A) represents distribution of cells following staining with the two dyes. Following infection with TMEV, most cells move rapidly into the quadrant that denotes secondary necrosis/necrosis.

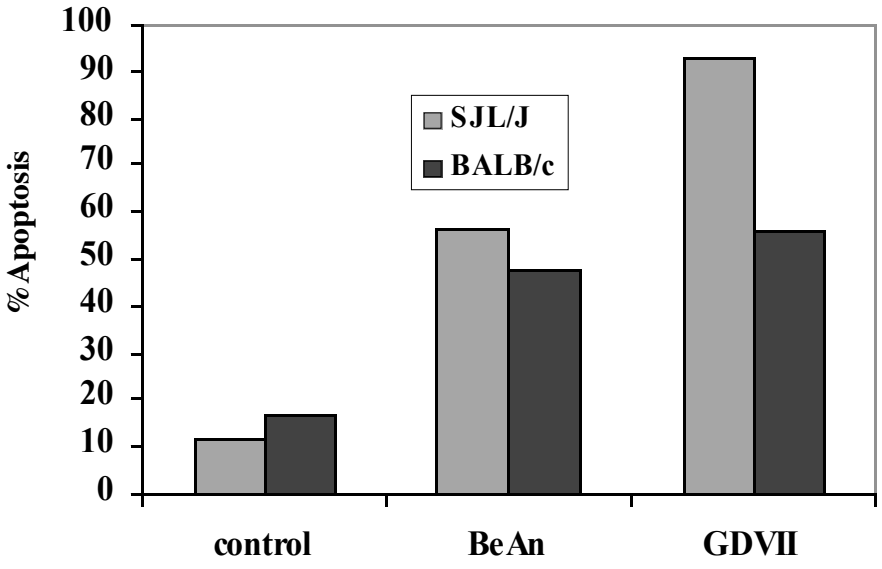

FIG. 7. Annexin V staining of TMEV-infected CVE (48h). SJL/J and BALB/c CVE were infected with BeAn and GDVII at MOI of 1 for 48h. Note that for BeAn infected CVE, more cells are apoptotic compared to 24h. GDVII was more apoptotic as compared to BeAn. The above figure is a representative of three independent experiments.

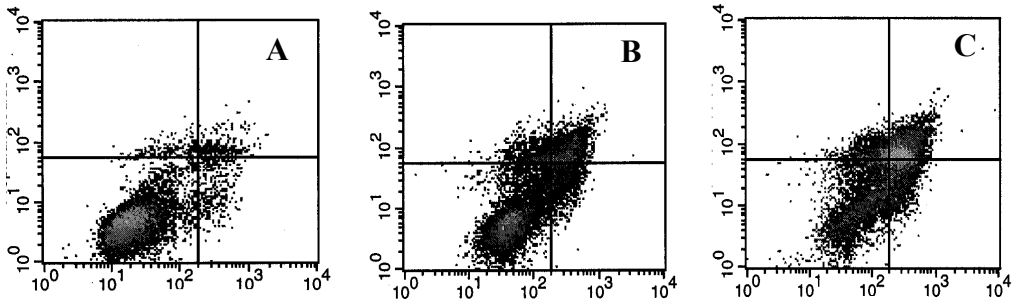

FIG. 8. Flow cytometry analysis of TMEV-infected BALB/c CVE (24h). BALB/c CVE were either mock-infected (B) or infected with BeAn (C) or GDVII (D) at MOI of 1 for 24 h and stained with Annexin V and PI as described in materials and methods. (A) represents distribution of cells following staining with the two dyes. Following infection with TMEV, most cells move rapidly into the quadrant that denotes secondary necrosis/necrosis.

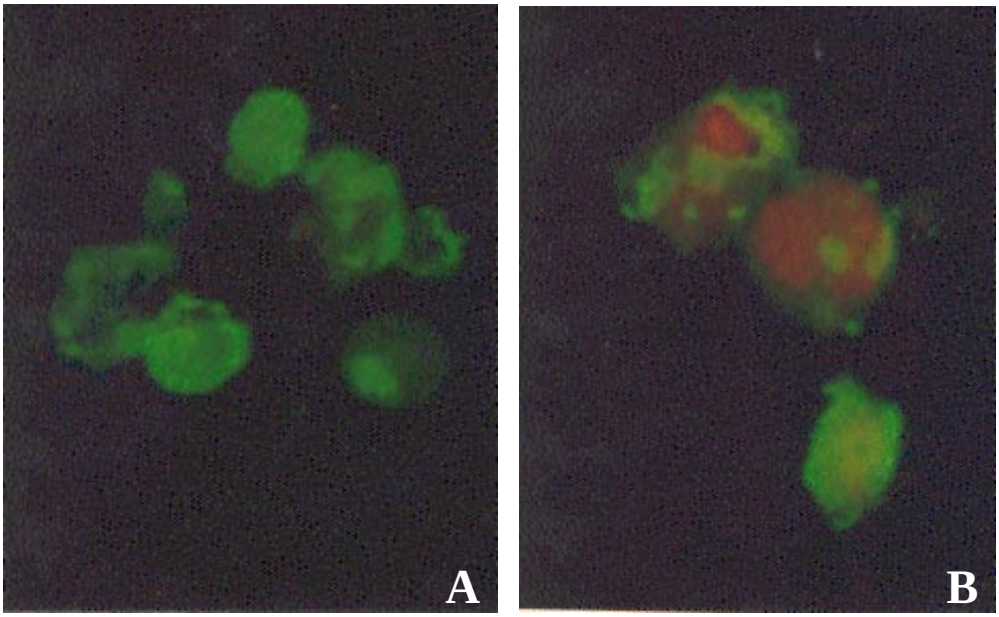

FIG. 9. Annexin V and PI staining of CVE infected with TMEV. Photomicrographs of SJL/J CVE infected with BeAn at MOI of 1 for 24h pi, sorted by Laser scanning cytometer. (A) shows cells in the initial stages of apoptosis that stain with Annexin V alone. (B) shows cells in the late stage of apoptosis, possibly indicating secondary necrosis, that stain with both Annexin V and PI.

Hoechst, caspase-3 and mitochondrial activity staining following infection of CVE with BeAn and GDVII

The morphology of apoptotic cells was further confirmed using Hoechst staining. SJL/J and BALB/c CVE were grown on cover slips and at the appropriate time points post-infection, the cells were washed and stained with Hoechst stain, caspase-3 stain and TMRM. Figure 10 indicates, increasing induction of apoptosis by BeAn and GDVII in SJL/J CVE over time. Mock-infected CVE displayed very minimal signs of apoptosis only at 48h. Hoechst staining indicative of nuclear involvement in the development of apoptotic process can be seen by 18h p.i. Apoptotic nuclei can clearly be demonstrated by nuclear condensation. The percentage of apoptotic nuclei in both CVE infected with BeAn and GDVII increases over time and ultimately clumps of cells that have been dislodged due to cytopathic effects (CPE) can be seen comprising apoptotic nuclei (Fig. 11). Thus, we can infer that CPE and apoptosis may be closely linked. TMRM staining indicates strong mitochondrial activity prior to induction of apoptosis as determined by Hoechst staining (Fig. 12). This starts at 12h p.i and is reduced by 24h p.i when apoptotic nuclei can be demonstrated. Caspase-3 activity can be demonstrated as early as 3h p.i following viral infection of the CVE. No caspase-3 activity was observed in mock-infected cells. Caspase-3 activity appears to be one of the earliest apoptosis-related events in CVE infected with TMEV (Fig. 13). GDVII infection did not cause heightened activity of caspase-3, or show increased Hoechst staining as compared to BeAn infected CVE.

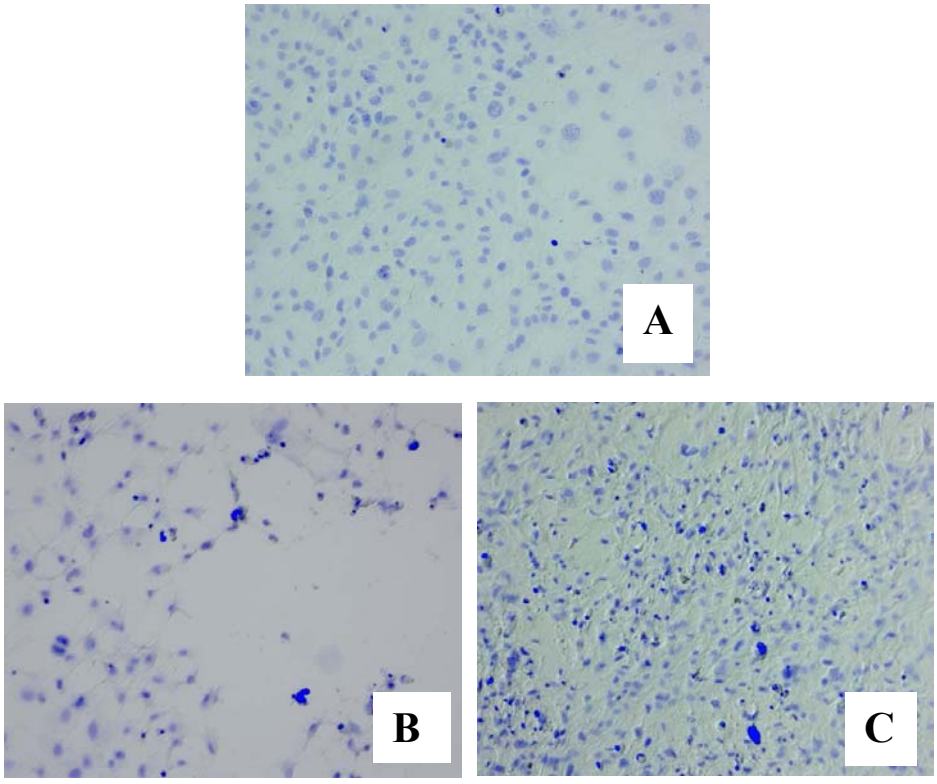

FIG. 10. Morphological changes in TMEV-infected SJL/J CVE. SJL/J CVE were grown on glass cover-slips and mock-infected (A) or infected with BeAn (B) or GDVII (C) at MOI of 1 for 24h pi and stained with Hoechst 33342 to observe nuclear changes. Nuclear condensation and cell shrinkage can be clearly observed in BeAn and GDVII infected CVE.

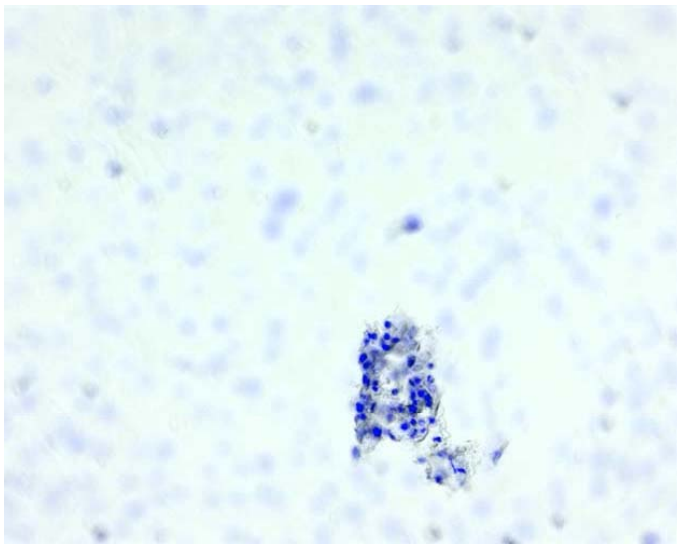

FIG.11. Induction of apoptosis and cytopathic effects are correlated. SJL/J CVE infected with BeAn at MOI of 1 for 24h was stained by Hoechst 33342. Clumps of cells dislodged due to cytopathic effects of the virus contained numerous apoptotic nuclei.

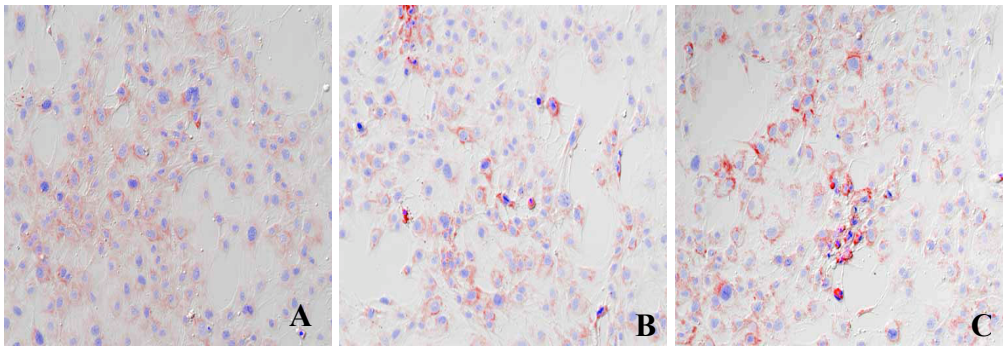

FIG. 12. Mitochondrial activity changes in TMEV-infected SJL/J CVE. SJL/J CVE were grown on glass cover-slips and mock-infected (A) or infected with BeAn (B) or GDVII (C) at MOI of 1 for 18h pi and stained with TMRM and Hoeschst 333 42 to observe mitochondrial activity changes as well as nuclear changes. Mitochondrial activity (red) can be observed prior to nuclear changes in TMEV infected CVE. A few cells showing nuclear condensation (blue) can be observed in BeAn and GDVII infected CVE. .

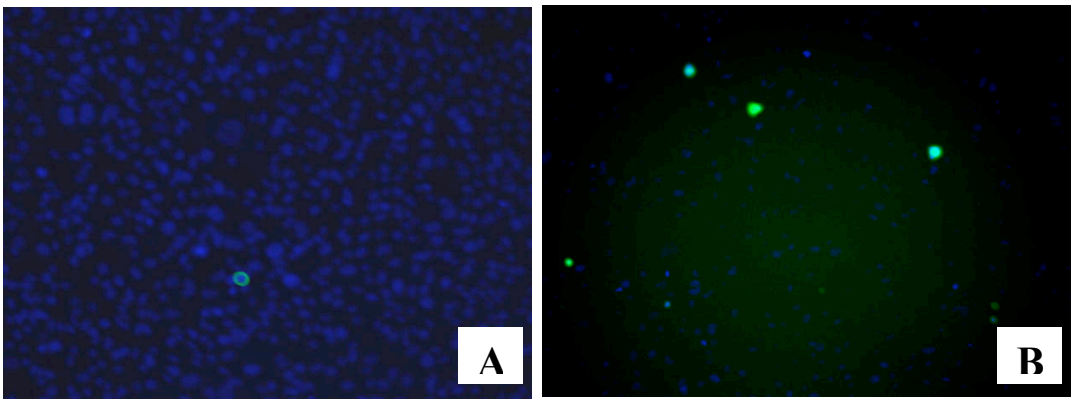

FIG. 13. Caspase-3 activity in TMEV-infected SJL/J CVE. SJL/J CVE were grown on glass cover slips and mock-infected (A) or infected with BeAn at MOI of 1 for 3h and stained with Rhodamine 110 to detect Caspase-3 activity. There was an increase in Caspase-3 activity only in BeAn infected CVE as shown by the green fluorescence.

DNA fragmentation assay

DNA oligonucleosomal laddering is one of the hallmarks of apoptosis and is one of the ultimate steps in the death of the cell by apoptosis. Figure 14 shows that BeAn and GDVII infected SJL/J CVE show DNA fragmentation at 24h and 48h. There is no DNA fragmentation observed in mock- infected CVE. It is interesting to note that in addition to DNA laddering a smearing pattern is observed, indicating possibly secondary necrosis.

Electron microscopic analysis of apoptosis

Induction of apoptosis in SJL/J and BALB/c CVE infected with BeAn and GDVII was observed ultramicroscopically (Figs. 15 and 16). Electron microscopic analysis confirms the induction of apoptosis in CVE infected with Theiler's virus. The CVE were infected with BeAn and GDVII at MOI of 1 for 24h. At 24h, mock-infected CVE do not show considerable signs of apoptosis. Uninfected BALB/c CVE show some initial signs of apotosis with minimal nuclear margination. This observation corresponds to the Annexin V data, which indicates BALB/c CVE show slight signs of apotosis upon serum deprivation. Infection of both CVE with BeAn shows classical signs of apoptosis as is indicated by surface blebbing, nuclear margination and condensation and loss of organelles. Infection with GDVII shows heightened signs of apoptosis with nuclear condensation, loss of organelles and even appearance of apoptotic bodies. This shows signs of secondary necrosis, which correspond to the data from the Annexin assay.

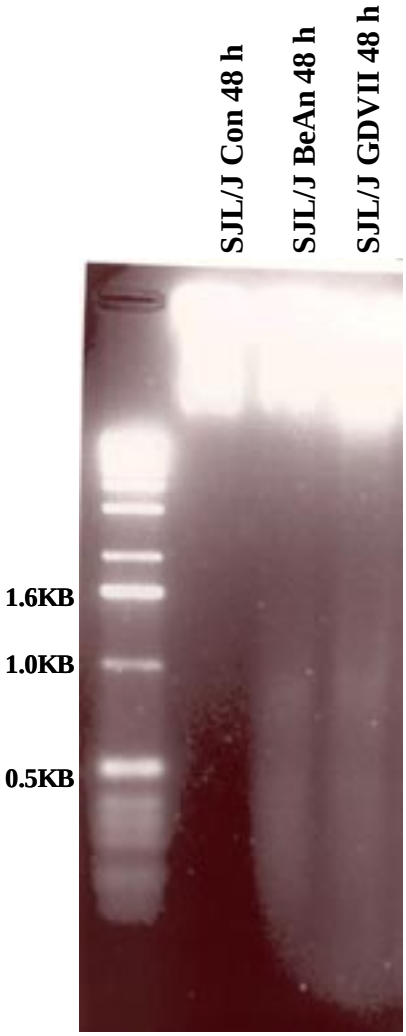

FIG. 14. DNA laddering in TMEV-infected SJL/J CVE. SJL/J CVE were either mock infected or infected with BeAn or GDVII at MOI of 1. At 48h pi, DNA was extracted, processed and subsequently 1 μg of extracted DNA was analyzed by electrophoresis in a 1.5% agarose gel as described in materials and methods. DNA laddering was observed only in BeAn and GDVII infected CVE.

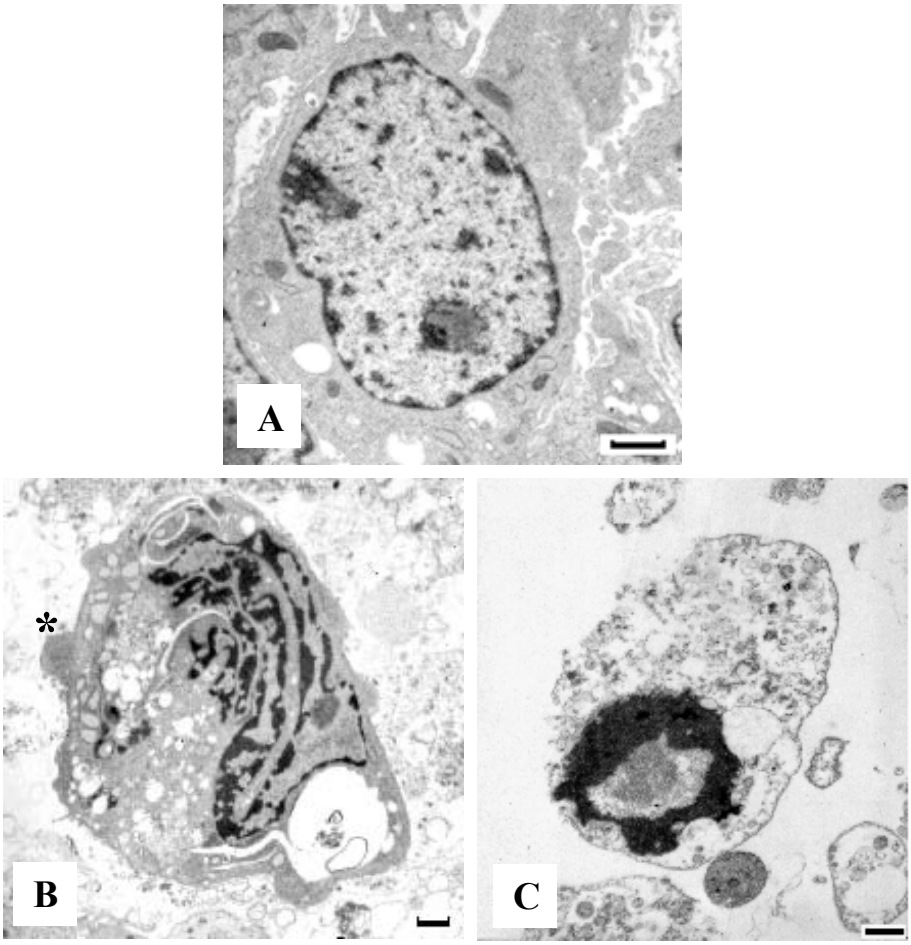

FIG. 15. Ultramicroscopic details of TMEV-infected SJL/J CVE. Electron micrographs of mock-infected SJL/J CVE (A) (5000Xmagnification) and SJL/J infected with BeAn (B) (6300X magnification) or GDVII (C) (5000X magnification) at MOI of 1 for 24 h pi. Cells were fixed and processed as described in materials and methods. Nuclear and cellular abnormalities, characteristic of apoptosis, are observed in CVE infected with BeAn and GDVII only. These changes include nuclear condensation, loss of organelles and surface blebs (*). Bar represents 1μm.

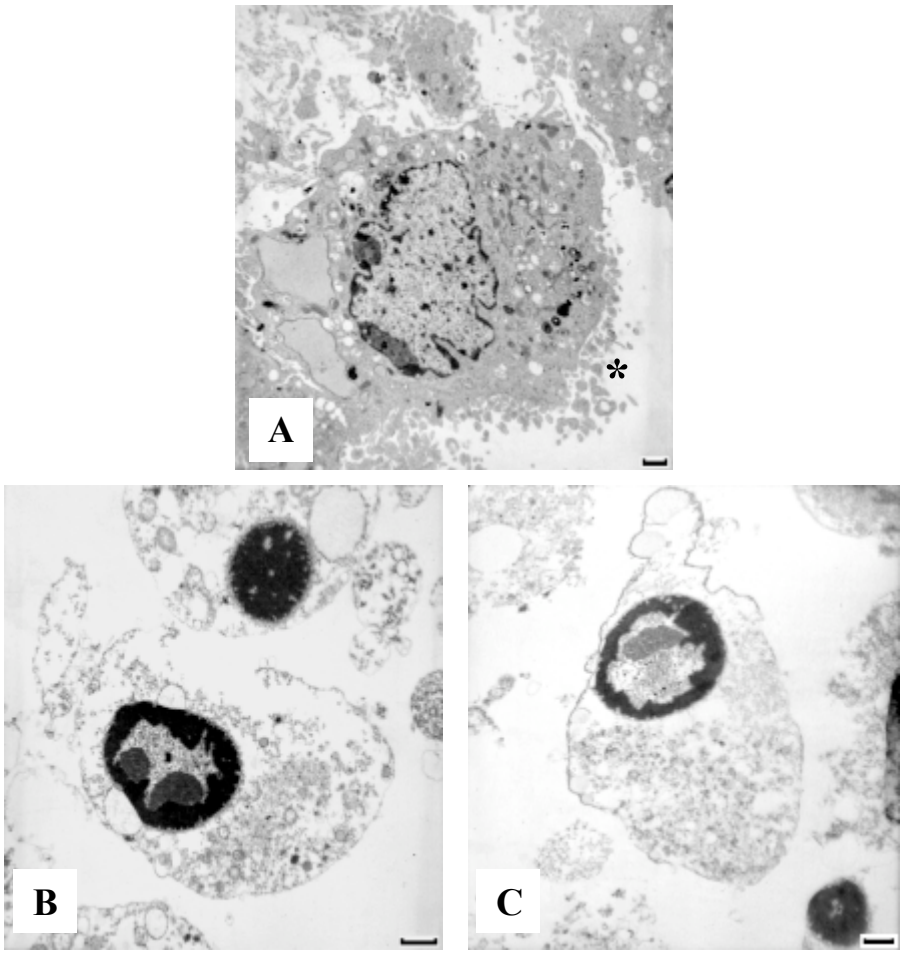

FIG. 16. Ultramicroscopic details of TMEV infected BALB/c CVE. Electron micrographs of mock-infected BALB/c CVE (A) (6300X magnification) and BALB/c CVE infected with BeAn (B) (5000X magnification) or GDVII (C) (5000X magnification) at MOI of 1 for 24h pi. Cells were fixed and processed as described in materials and methods. Nuclear and cellular abnormalities characteristic of apoptosis are observed in CVE infected with BeAn and GDVII only. These changes include nuclear condensation, loss of organelles and surface blebs (*). Mock-infected BALB/c CVE appear to undergo some level of apoptosis following serum deprivation. Bar represents 1 m.

DISCUSSION

Experiments described in this report demonstrate the induction of apoptosis in cloned mouse CVE derived from two different strains of mice following infection with TMEV. This is the first report of apoptosis in CVE following infection with TMEV. Apoptosis induction was similar in both types of CVE. Theiler's virus from both virulence groups have been reported to induce apoptosis in a variety of cells both *in vivo* and *in vitro*. Whether the induction of apoptosis is important in the pathogenesis of the virus has yet to be determined. Following intracranial infection with TMEV, a variety of cells including neurons (10, 241), oligodendrocytes (241), macrophages and astrocytes have been postulated to carry a major viral load and to undergo apoptosis. TMEV is capable of inducing apoptosis in both permissive as well as restrictive cells. GDVII has been shown to induce more apoptosis than the TO strains, thus indicating that neurovirulence can be associated with increased apoptosis. This could account for the fatal polioencephalitis following GDVII infection intracranially. The role for anti-apoptotic proteins has been postulated in TO strains, which could lead to persistence. L* - an out of frame protein is found in DA and not in GDVII and it has been reported to have anti-apoptotic features (63).

In our study, we have shown that infection of CVE from two genetically different strains of mice, with TMEV, resulted in the induction of apoptosis. This induction of apoptosis was dose dependent and required replicating virus. Increasing the MOI of the virus showed increasing CPE and increasing effect on apoptosis suggesting a strong link between the two. Further, Hoechst staining of CVE confirmed this finding. Floating

cells that were dislodged by CPE showed clumps of highly apoptotic cell. Hence, similar to other viruses, we can infer that CPE in CVE is directly related to apoptosis.

UV-inactivation of the virus did not result in apoptosis indicating that apoptosis was not receptor mediated and there did not appear to be a role for death related receptors. Similar findings have been reported with TMEV infection of macrophages, where UV-inactivated virus failed to induce apoptosis. Complete removal of serum from the medium signaled for some apoptosis, but the low percentage of serum (1%) during viral infection procedures did not greatly affect induction of apoptosis by TMEV. It is interesting to note that BALB/c CVE were more susceptible to apoptosis following serum deprivation than SJL/J CVE. This could also be clearly recognized by electron microscopy. Following serum deprivation, BALB/c CVE shows some initial signs of apoptosis.

Earlier studies by Welsh (251) have shown that CVE are permissive to TMEV and resulting viral titers are comparable to those from BHK-21 cells. GDVII showed a very slight increase in CPE compared to BeAn. Our studies in this report confirm these findings. CVE are permissive to TMEV infection and yield infectious progeny following infection for 8hr. An MOI of 1 was selected for our studies since this low infection would be more physiological. In addition, MOI of 1 seemed the appropriate MOI as it did not rapidly cause apoptosis and allowed for the observation of onset of apoptosis over time. At this MOI, there were signs of apoptosis at 18h p.i. There were very few signs of apoptosis in BALB/c or SJL/J CVE prior to 18h p.i. and this was confirmed by Annexin V, Hoescht staining and EM. However, there is caspase

activation as early as 3h and also mitochondrial activation as early as 3h. The results from Hoechst staining coupled with mitochondrial staining, strongly suggests the mitochondrial activation of CVE prior to induction of apoptosis. Apoptosis is an energy requiring procedure and the results confirm this. Following induction of apoptosis there is decreased mitochondrial activity.

A number of other neurotropic viruses replicate in the CVE and can mediate damage. Infection of CVE may promote damage both directly as well as indirectly. Some of the viruses that can replicate productively in endothelial cells of the CNS both in vivo and in vitro are SIV (144), Measles virus (41), Canine distemper virus (14), Semliki Forest virus (SFV) (228). In the case of Theiler's virus, oral infection of neonatal mice suggests route of entry of TV is through the BBB (73).

TMEV is a natural enteric pathogen of mice and the incidence of spontaneous paralysis is low. In the natural course of infection, TMEV replicates in the gastro-intestinal tract of mice and it occasionally gains access to the CNS where it sets up a persistent infection. It has been hypothesized that BBB could serve as an important entry site for the virus into the CNS. Alterations in the BBB could allow the entrance of inflammatory cells into the CNS, which would then mediate the damage associated with TVID. The disruption of the BBB seems to be a crucial event in the development of CNS inflammatory disorders like MS. Both in MS and TVID, the BBB is disrupted during the early stage of the disease and BBB dysfunction has been associated with relapses in MS. Very recent reports confirm the involvement of the BBB. The leakage of serum proteins into extravascular spaces in the brain has been reported by many studies

(28, 123), indicating BBB dysfunction. There is increased expression of adhesion molecules such as ICAM-1 and VCAM-1 on patients with active MS (64, 65), (76). In a recent report there is some speculation about the role of apoptosis of CVE in the development of the disease. Minagar et al. (159), report that there is an increase in the levels of plasma microendothelial particles in MS. These have been hypothesized to be vesicles of endothelial cells that have been shed by apoptosis. In addition, in MS and in TVID, lymophocytic cuffing is observed around post-capillary venules and this suggests that the main route of cellular migration may occur across the BBB. Viral antigens have been detected early in TVID in the CVE (259) and this infection of CVE may play a very crucial role in the development of the disease. It is also interesting that many of the viruses that have been implicated in the etiology of MS or shown to cause demyelination in animals also infect CVE. CVE possess many specialized receptors that may function as viral receptors. Infection of CVE could result in many outcomes such as upregulation of various molecules involved in lymphocyte adhesion, or the direct infection of CVE could result in apoptosis leading to a breach in the BBB.

Apoptosis of CVE has been suggested to be an important factor in the development of disease in HIV-1 (33, 220), SIV (4) and Influenza A virus (85) infection of the CNS. Apoptosis of endothelial cells of other organs have been reported in conditions such as acute lung damage, systemic sclerosis, and as a complication of non-ionizing radiation. In culture, apoptosis of CVE can be induced by bilirubin (5) and oxy-hemoglobin (152, 153).

Our findings demonstrate that Theiler's virus induces of apoptosis *in vitro* in CVE. The induction of apoptosis is dependent on the MOI of the virus, involves the activation of caspase-3 and also there is a correlation between CPE and the ability of the virus to induce apoptosis. Like in other cell lines, GDVII is capable of inducing increased apoptosis in CVE as compared to BeAn. These findings may hold importance in terms of the pathogenesis of the virus and CNS invasion. Induction of apoptosis in CVE *in vivo* following systemic circulation could provide a route of entry for the virus viruses as well as inflammatory cells into the CNS.

CHAPTER III

CALPAIN INVOLVEMENT IN TMEV-INDUCED APOPTOSIS OF CVE

INTRODUCTION

Apoptosis or programmed cell death is a form of physiological cell death characterized by the sequential activation of a series of proteins that ultimately leads to the death of the cell. The hallmarks of apoptosis include DNA condensation in the nuclei, DNA fragmentation at the nucleosome linkage regions, plasma membrane blebbing, cell shrinkage and the ultimate formation of apoptotic bodies, which are phagocytosed by macrophages without eliciting an inflammatory response (55, 216, 246). Apoptosis is a process required for normal development of multicellular eukaryotes and is required by processes such as elimination of self-reactive T cells in the thymus. However, the inappropriate initiation of apoptosis is thought to contribute to the etiology of several pathologies, including neurodegenerative disorders and autoimmune diseases, such as Type 1diabetes (150).

A variety of viruses are capable of provoking apoptotic cell death. Neurotropic RNA viruses that have been associated with apoptosis *in vivo* include human immunodeficiency virus (HIV) (186), dengue virus (49), sindbis virus (128), reovirus (168), poliovirus (67), and Theiler's murine encephalomyelitis virus (TMEV) (241). Other members of *Picornaviridae* family that induce apoptosis in cell culture include

coxsackie B3 virus (31), hepatitis A virus (22). Poliovirus is unique in its ability to induce as well as inhibit apoptosis (238). Most RNA viruses do not carry anti-apoptotic genes, with the exception of poliovirus, and they usually induce apoptosis in the cells they infect and escape deleterious effects by rapid multiplication.

Theiler's murine encephalomyelitis virus (TMEV) is a RNA virus belonging to the *Picornavirdae* family. Like other members of this family, it is also capable of inducing apoptosis. TMEV is a commonly used animal model for the human disorder- Multiple Sclerosis (MS). In mice, following intra-cranial (i.c) injection, the virus has been shown to induce apoptosis in a variety of cell types including neurons, oligodendrocytes, and astrocytes (241). TMEV can induce apoptosis *in vitro* in differentiated macrophages (92), simian kidney cells - BSC-1 cells (95) and neurons (10, 241). The neurovirulent strain of the virus – GDVII, causes fatal encephalitis in all strains of mice is postulated to act through the rapid induction of apoptosis in the cell types infected. The role for an anti-apoptotic protein- L* protein has been implied in the persistence of the TO strains of TMEV (63). The pathway for induction of apoptosis following TMEV infection has not been completely understood, though a role for TRAIL (94) has been suggested in macrophages infected with TMEV *in vitro*. Caspases are central to most apoptotic pathways following infection with viruses, but several other proteases can also play a role. This includes a calcium-induced neutral cysteine protease, calpain, which has been reported to be unregulated in MS (17, 223) as well as the autoimmune model for MS-experimental allergic encephalitis (EAE) (221, 222, 224).

The route of entry of TMEV into the central nervous system (CNS) following natural enteric infection is uncertain. One of the proposed portals of entry includes invasion of the blood-brain barrier (BBB) by infection of the cerebrovascular endothelial cells (CVE) (119, 250, 251, 259). Several other viruses such as HIV 1 (33), Simian Immunodeficiency virus (SIV) (4), measles virus (41), Semliki forest virus (SFV) (56) enter the CNS through this route. HIV (220) and SIV (4) have been reported to induce apoptosis in the CVE and thus allow for the breakdown of the BBB. TMEV is capable of infecting CVE *in vitro* (119, 251) and also inducing MHC II (251) expression thus suggesting a role for these cells in the invasion of the CNS by TMEV.

Previous data demonstrate the ability of both neurovirulent and neuropersistent strains of TMEV to induce apoptosis in cloned mouse CVE derived from TVID-susceptible (SJL/J) as well as TVID-resistant (BALB/c) strains of mice. Like other picornaviruses, TMEV induces cytopathic effects in the CVE and previous work indicates that the induction of apoptosis and cytopathic effects may be related. There appears to be a dose dependent relation for the virus to induce apoptosis and like many other viruses, UV-inactivation does not induce apoptosis in CVE.

The aim of the following study was to delineate the pathway of apoptosis in CVE following infection with TMEV *in vitro*. The pro-apoptotic protein profile was studied and questions regarding difference in the abilities of GDVII and BeAn to induce apoptosis were addressed. In addition, a role for Calpain in the induction of apoptosis in CVE following TMEV infection was studied.

MATERIALS AND METHODS

Virus

The BeAn 8386 strain and GDVII were obtained from Dr. Howard L. Lipton (Northwestern University, Evanston, IL). The virus was grown in BHK-21 cells, and the culture supernatant containing infectious virus was aliquoted and stored at -70C before use. The viral titer was determined by plaque assay on BHK-21 cells (206).

Cell culture

CVE that have been isolated from brain microvessels of either TVID-susceptible (SJL/J) or resistant (BALB/c) mice were used in these experiments. These cells were isolated and cloned by limiting dilution as described previously (213). These CVE remain diploid and maintain their differentiation markers in culture. Frozen aliquots were thawed and grown in Iscove's Modified Dulbecco's Medium (IMDM)(GIBCO BRL, New York, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 2mM glutamine (GIBCO BRL, New York, NY), 100units/ml Penicillin and 100 g/ml Streptomycin (GIBCO BRL, New York, NY) at 37°C and 4%CO_2.

Infection of CVE

SJL/J and BALB/c CVE were grown in IMDM supplemented with 10% FBS for growth and 1% FBS for post inoculation maintenance of cell cultures. For infection with virus, the CVE were grown to confluence in T 25 flasks and infected with BeAn and GDVII strains of Theiler's virus at a MOI of 1. The virus were allowed to adsorb for 45 min at room temperature and were subsequently washed with IMDM and further incubated in IMDM with 1% FBS. Control cells were treated similarly but without the addition of

the virus. Cell suspensions were prepared by Trypsin/EDTA (GIBCO BRL, New York, NY) treatment at the required time points and utilized for the respective assays.

UV-inactivation of the viruses

Inactivation of both BeAn and GDVII was carried out by exposing previously titered virus infected BHK cell lysates to the UV illuminator lamp (1330 W/cc) at a distance of 13cm for 30 min. UV-inactivation of the virus was confirmed by performing plaque assay.

RNase Protection Assay (RPA)

RNA was isolated from the infected cells and controls using the Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The RiboQuant multiprobe RNase protection assay (RPA) (Pharmingen, San Diego, CA) was used to analyze the RNA expression of caspase-8, FASL, FAS, FADD, TRAIL, TRADD and RIP, using the mAPO-3 template from Pharmingen. The probe was synthesized using [^{32}P] UTP.

Western blot analysis of apoptosis related proteins

The expression of pro-apoptotic proteins was assessed by Western blotting. Virus infection of SJL/J and BALB/c CVE were done as previously described. After the respective time points, the cells were lysed in a buffer containing 50 mMTris-HCl (pH 7.5), 150mM sodium chloride; and 0.5%NP-40. Lysates were micro-centrifuged at 4°C for 10 min, and the supernatants were stored at -20°C. Protein concentration was determined by the Bio-Rad protein assay. Equal amounts of the total protein (20 g) were then resolved on an 8-15% polyacrylamide gel depending on the requirement and

transferred to a Hybond-C nitrocellulose membrane (Amersham Biosciences, England) by semi-dry blotting. The membrane was blocked with Tris-buffered saline containing 5% non-fat dry milk and 0.02%Tween 20. This was then incubated with affinity purified rabbit anti-active caspase-3 monoclonal antibody (1:2000)(BD Pharmingen, San Diego, CA); purified mouse anti-human PARP monoclonal antibody (1:2000) (BD Pharmingen, San Diego, CA); mouse monoclonal anti-FAS antibody (1:500) (Transduction Laboratories, Lexington, KY), mouse monoclonal RIP antibody (1:500) (Transduction laboratories, Lexington, KY), mouse anti-calpain 1 Large subunit monoclonal antibody (1:1000)(Chemicon, Temecula, CA), anti-□-fodrin monoclonal antibody (1:1000)(ICN, Aurora, OH). The expression of these proteins was detected by ECL according to the manufacturer's (Amersham Biosciences, England) instructions. Jurkat cell total protein lysate (Biomol, Plymouth meeting, PA) served as the size-fractionated positive control.

Intracellular free calcium assay

SJL/J CVE were grown to 70% confluence in 6-well plates and later infected with BeAn and GDVII at MOI of 1. Following the appropriate incubation time post-infection, the plates were washed twice with IMDM. The cells were then incubated in 1ml IMDM containing cell permeable fluo 3-acetoxymethyl ester (fluo-3) (Molecular Probes, Eugene, OR) as fluorescent calcium indicator at a final concentration of 10□M. The plates were then incubated at 37C with 5% CO_2 for 1h. The wells were then washed twice with IMDM and replaced with 1ml IMDM. The plates were then read on a fluorometer with fluorescence for fluo 3-acetoxymethyl ester measured with filters for

excitation at 485 nm and emission at 538 nm. Relative cell counts were performed using staining with Janus Green. Briefly the procedure for staining was as follows: The cells were washed twice with IMDM and incubated in 1ml ethanol for 90 sec. Following drying for 3 min, 1 ml of Janus Green stain was added to the cells and incubated for 1 min. The cells were then washed twice with 1X PBS. 500µl ethanol and 500µl PBS were added to each well and the plate was read on the fluorometer with absorbance at 645nm.

RESULTS

Caspase-3 activation following TMEV infection of SJL/J and BALB/c CVE

Following infection of SJL/J and BALB/c CVE with BeAn and GDVII over time points 12h, 18h and 24h post-infection, cleavage of the zymogen caspase-3 to its pro-enzyme form can be observed (Fig. 17). The zymogen is cleaved over time following infection and by 24h, the cleavage is complete. Earlier results demonstrate that caspase-3 activity is started as early as 3h.

PARP cleavage following TMEV infection

Poly (ADP) ribose polymerase (PARP) is a nuclear substrate for caspase-3 and several other proteases including calpain. Active PARP exists as a 119kDa protein in the nucleus and cleavage of PARP is downstream of caspase-3 activation. Following cleavage by caspase-3, PARP is degraded to an 89kDa protein (126). However, recent reports indicate that PARP can be degraded by other proteases such as calpain. Calpain degradation of PARP yields fragments, which are 70kDa to 40kDa (211, 217). The cleavage pattern shown in fig. 18, indicates cleavage of PARP by proteases other than

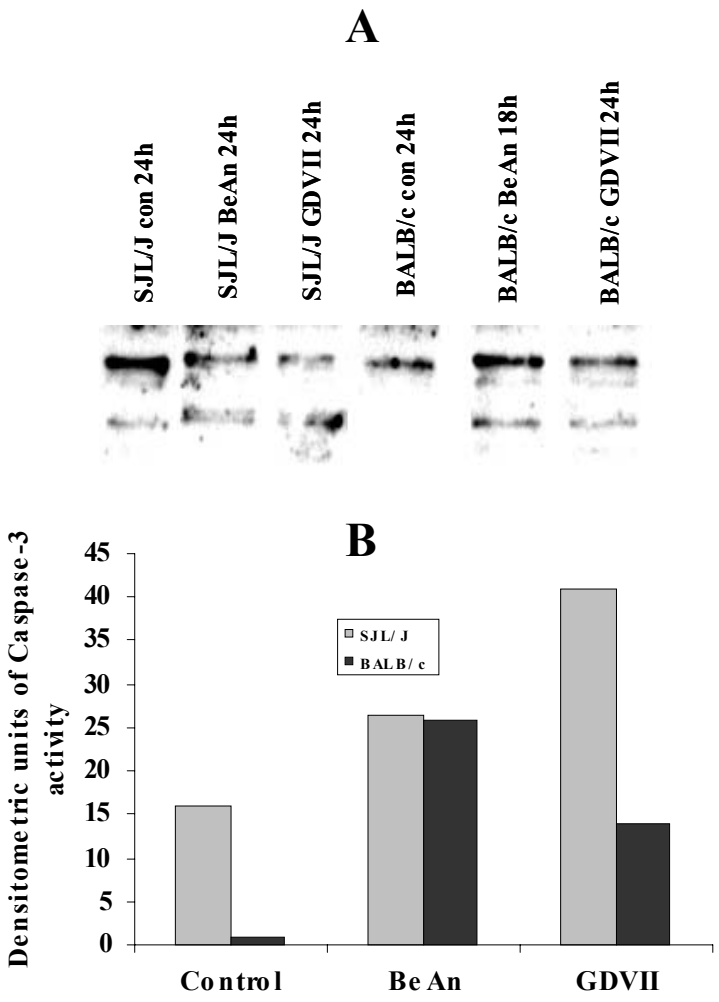

FIG. 17. Caspase-3 activation in TMEV-infected CVE. Pro-caspase (32kDa) is activated by its conversion to active caspase-3 (17kDa). SJL/J and BALB/c CVE were either mock infected or infected with BeAn or GDVII at MOI of 1 for the indicated times and immunoblotted with antibody to caspase-3 (A). (B) indicates densitometric analysis of caspase-3 activity.

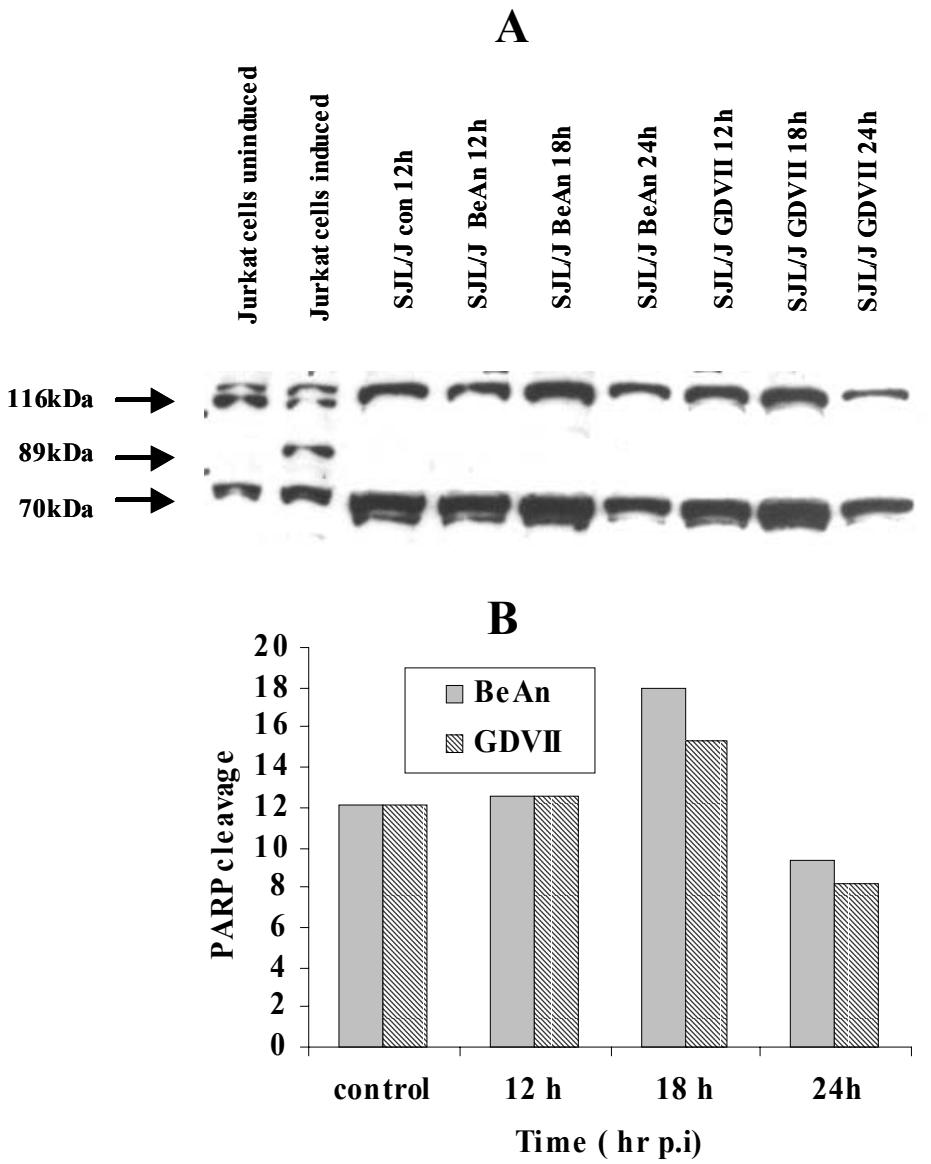

FIG. 18. PARP cleavage in TMEV-infected CVE. The 116kDa intact form of PARP is broken down to 89kDa only in treated Jurkat cells. Following infection of CVE with TMEV, PARP is broken down to 70kDa cleavage fragments. SJL/J CVE were either mock-infected or infected with BeAn or GDVII at MOI of 1 for the indicated times and immunoblotted with antibody to PARP (A). (B) indicates the densitometric analysis of PARP cleavage.

caspase-3. Mock-infected CVE do not undergo PARP degradation and PARP degradation in CVE infected with BeAn or GDVII appears to increase at 18h post-infection. By 48h, PARP is totally degraded and even the 70kDa protein in further degraded.

RNase protection assay for surface-related apoptotic molecules

RNA isolated from CVE infected with TMEV was analyzed for difference in mRNA expression in surface-related apoptotic proteins. Figure 19 indicates there is no upregulation of any of these genes following TMEV infection and this is confirmed by fig. 20. In the previous chapter we have shown that following infection with UV-inactivated virus, there was no induction of apoptosis suggesting apoptosis was an intrinsic effect and not the result of the involvement of death receptors. However, TRAIL has been suggested to mediate apoptosis in IFN-γ treated macrophages infected with TMEV (94).

Western blot analysis of Fas and RIP

To confirm the RPA findings, protein was isolated following TMEV infection of SJL/J and BALB/c CVE for various time points and the protein expression of these surface-related apoptotic proteins were determined. Interestingly, following TMEV infection, Fas expression appeared to be decreased (Fig. 21). Similarly, RIP expression was reduced compared to mock infected controls (Fig. 22). Results were comparable with both BeAn and GDVII. This was unique but confirmed the RPA finding that apoptosis was an intrinsic mechanism following TMEV infection of CVE *in vitro*.

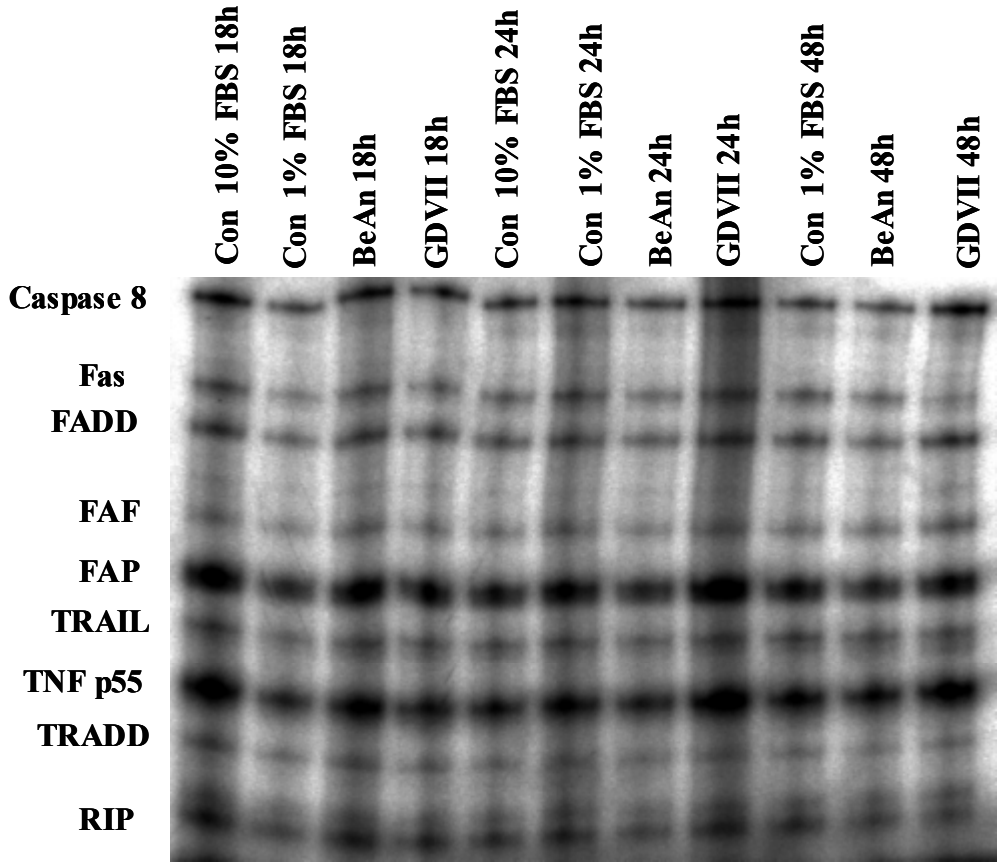

FIG. 19. RNase protection assay on SJL/J CVE with mAPO-3. The expression of death receptors and ligands is not altered following infection of SJL/J CVE with BeAn or GDVII. RNase protection assays with specific probes for caspase 8, Fas, FADD, FAF, FAP, TRAIL, TNFp55, TRADD and RIP genes were performed by using 10 ug total RNA extracted from SJL/J CVE after either mock infection or infection with BeAn and GDVII at MOI of 1 for the time points indicated. As internal controls, the mRNA levels of a ribosomal protein (L32) and GAPDH were simultaneously analyzed. As a control, RNA extracted from mock-infected SJL/J CVE was examined. To identify the protected bands, the probe without RNase treatment was applied as a positive control.

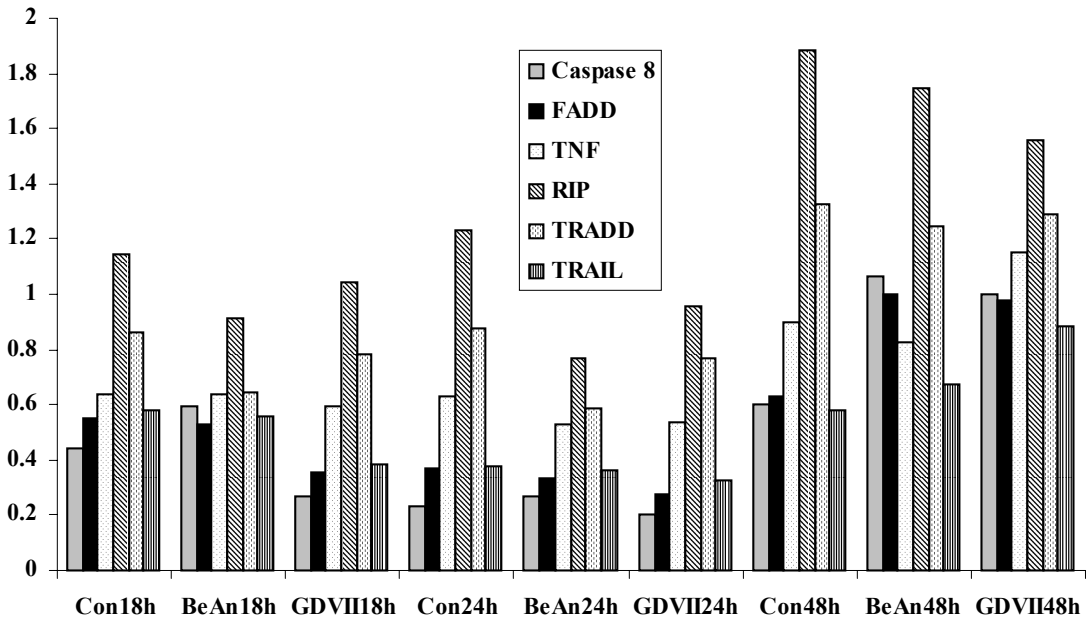

FIG. 20. Densitometric analysis of RNase protection assay. The expression of caspase-8, FADD, TNF, RIP, TRADD and TRAIL were compared in mock-infected CVE and SJL/J CVE infected with BeAn and GDVII over time points indicated. At 18h and 24h of infection with TMEV, the expression of these surface proteins related to apoptosis were below the levels of controls or at levels comparable to controls. However, at 48h infection with TMEV, there is a slight increase in the levels of expression of these surface proteins as compared to controls. But this change is not significant.

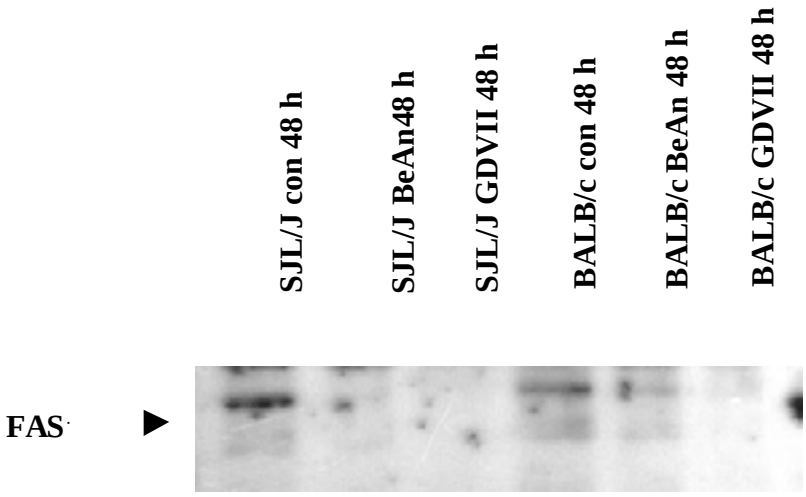

FIG. 21. FAS expression in TMEV-infected CVE. SJL/J and BALB/c CVE were either mock-infected or infected with BeAn or GDVII at MOI of 1 for the indicated times and immunoblotted with antibody to FAS. FAS (45kDa doublet) expression is reduced following TMEV infection.

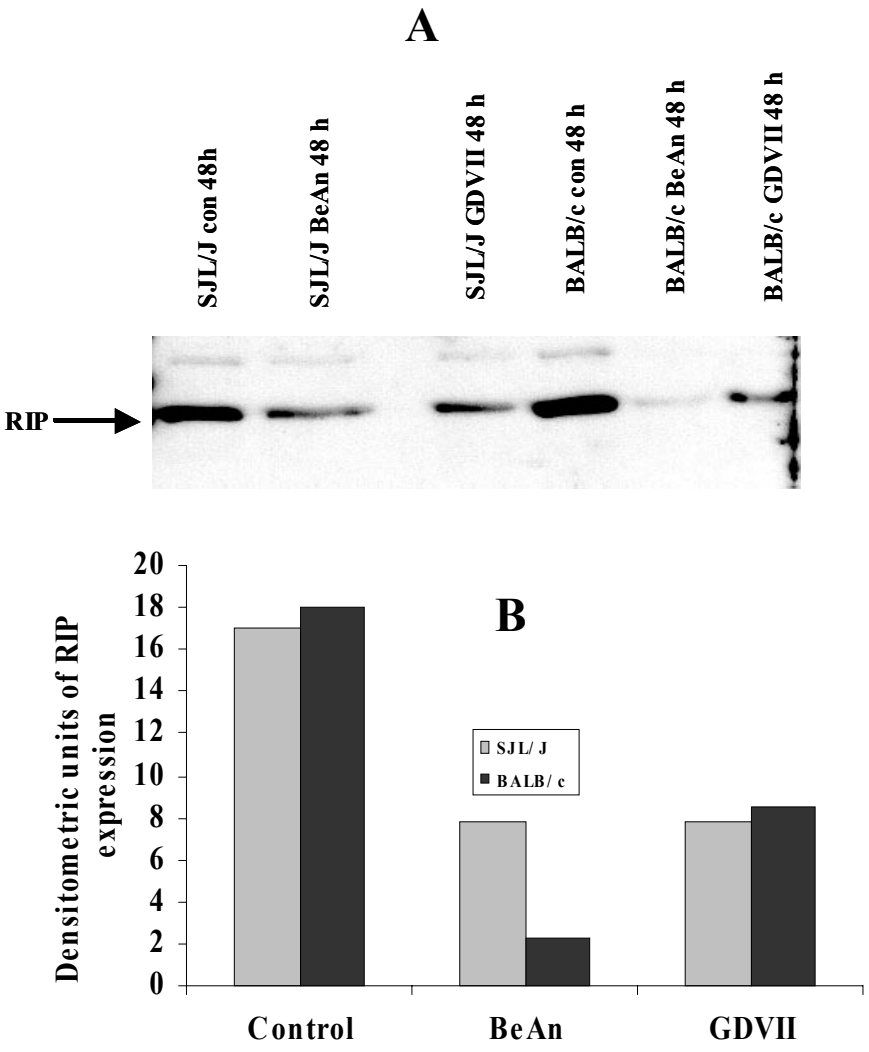

FIG. 22. RIP expression in TMEV-infected CVE. SJL/J and BALB/c CVE were either mock-infected or infected with BeAn or GDVII at MOI of 1 for the indicated times and immunoblotted with antibody to RIP(A). (B) indicates densitometric analysis of RIP expression. RIP expression is decreased following TMEV infection of the CVE for 48h.

Increase in free intracellular calcium following TMEV infection of CVE

SJL/J and BALB/c CVE were infected with BeAn and UV-inactivated BeAn for 12h and 24h periods and the changes in free intracellular calcium levels were determined using the calcium sensitive fluorescent dye - fluo 3. At 12h p.i, in both SJL/J and BALB/c CVE, there was no net increase in calcium levels as compared to mock-infected CVE. However, by 24h p.i, there was a sharp increase in calcium levels following infection as compared to mock-infected CVE (Figs. 23 and 24). This suggests a role for calcium in the apoptotic process and also for calpain, which is mediated through Ca^{2+}.

μ-calpain activity following infection of SJL/J and BALB/c with TMEV

Cleavage of μ-calpain from its 80kDa pro-enzyme form to the 76kDa zymogen form is indicative of its activation (154, 234). Following infection of SJL/J CVE and BALB/c CVE with BeAn and GDVII for 12h, 18h, and 24h, there was a definite increase in the pro-enzyme form of μ-calpain. The ratio of the 76/80 kDa form of μ-calpain was increased significantly reaching a maximum in 24h post-infection (Figs.25 and 26). The cleavage patterns were similar in BeAn and GDVII infected CVE. Mock-infected BALB/c CVE controls appeared to express very little amounts of the two forms and this may be related to the fact that BALB/c CVE is more susceptible to induction of apoptosis following serum deprivation as compared to SJL/J CVE. Interestingly, in SJL/J CVE, the cleavage of μ-calpain did not appear to start until 24h post-infection.

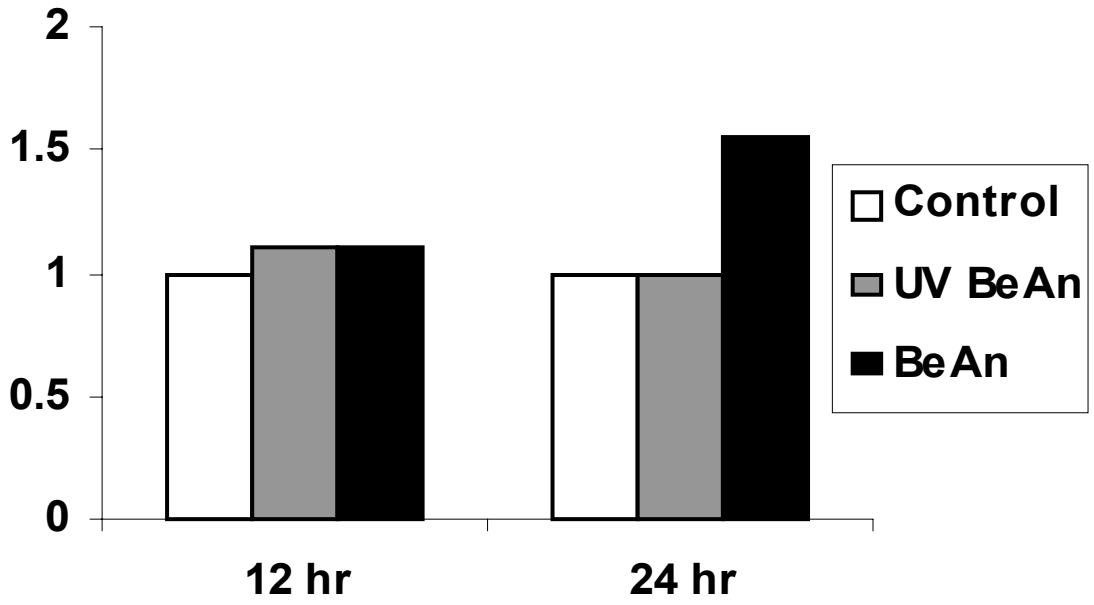

FIG. 23. Levels of free intracellular calcium in SJL/J CVE infected with BeAn. SJL/J CVE was either mock-infected or infected with BeAn at MOI of 1 for 12h and 24h. Calcium levels were measured by the fluorescent calcium indicator - fluo 3-acetoxymethyl ester. There is no increase in calcium levels at 12h following infection with BeAn. By 24h, there is a sharp peak in calcium levels in BeAn-infected SJL/J CVE.

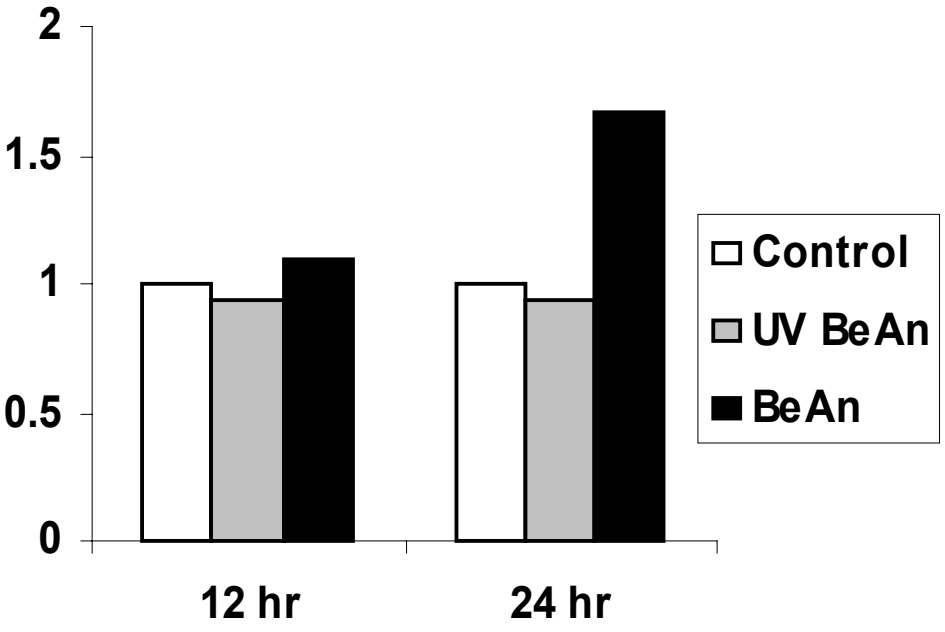

FIG. 24. Levels of free intracellular calcium in BALB/c CVE infected with BeAn. BALB/c CVE was either mock-infected or infected with BeAn at MOI of 1 for 12h and 24h. Calcium levels were measured by the fluorescent calcium indicator - fluo 3-acetoxymethyl ester. There is no increase in calcium levels at 12h following infection with BeAn. By 24h, there is a sharp peak in calcium levels in BeAn-infected BALB/c CVE.

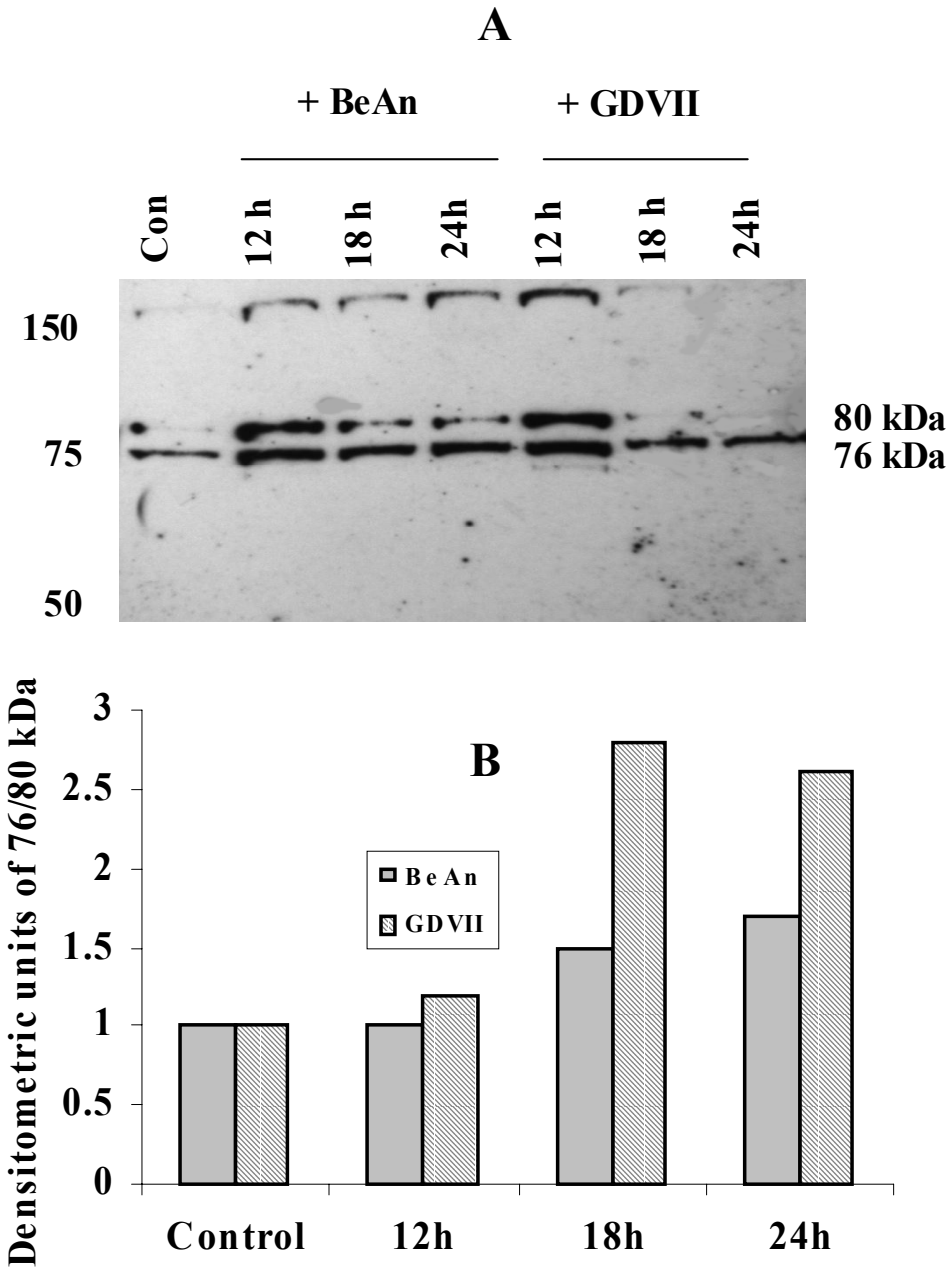

FIG. 25. Cleavage of μ-calpain in TMEV-infected SJL/J CVE. SJL/J CVE were either mock-infected or infected with BeAn or GDVII for the indicated times and immunoblotted with antibody to μ-calpain (A). (B) indicates that the ratio of 76/80 kDa of μ-calpain increased significantly in TMEV-infected CVE.

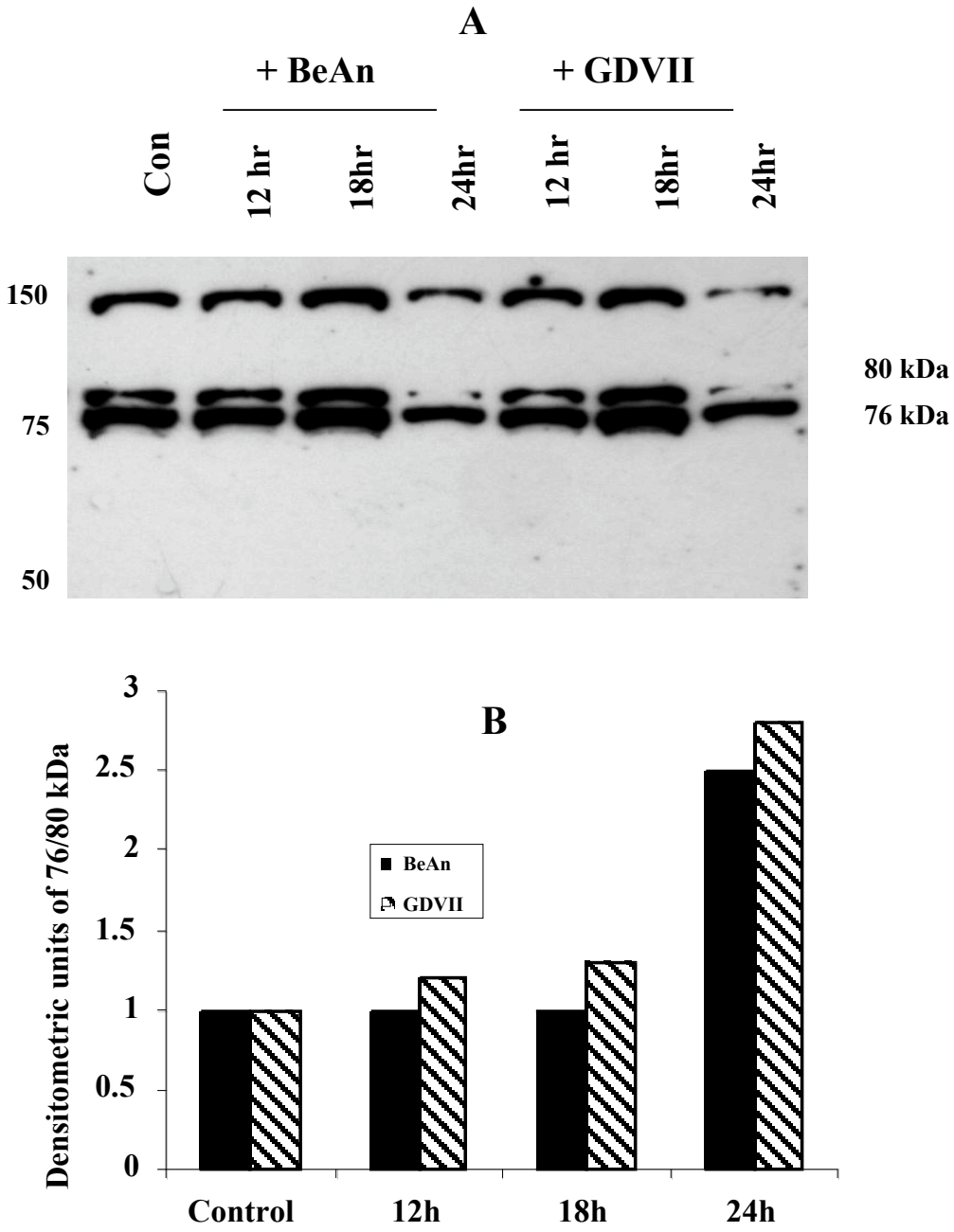

FIG. 26. Cleavage of □-calpain TMEV-infected BALB/c CVE. BALB/c CVE were either mock-infected or infected with BeAn or GDVII for the indicated times and immunoblotted with antibody to □-calpain (A). (B) indicates that the ratio of 76/80 kDa of □-calpain increased significantly in TMEV-infected CVE.

Fodrin cleavage infection of SJL/J and BALB/c with TMEV

Fodrin is a cytoskeletal component that is degraded by both caspase-3 as well as calpain. Degradation by either will give rise to characteristic breakdown products. 280kDa fodrin is broken down by caspase-3 to yield a 120kDa fodrin break down product (BDP) and by calpain to yield a 150/147 (doublet) kDa fodrin BDP (164) (Fig. 27). As is shown in fig. 28, there is increased fodrin cleavage following SJL/J CVE infection of BeAn and GDVII for 12h, 18h, and 24h. The major breakdown products are the calpain BDP and the maximum activity is seen at 18h p.i. Control cells show very little break down activity. There is also some low level of caspase-3 activity seen resulting in the 120kDa BDP. In addition, in BALB/c CVE (Fig. 29), interestingly there is a triplet banding pattern reported by other authors seen in the calpain related BDP (16, 196). This banding pattern has not been explained. In BALB/c CVE, there is very little caspase-3 breakdown of fodrin. Earlier experiments had determined that UV-inactivated virus was not capable of inducing apoptosis in CVE. This was further tested by Western blot analysis for fodrin cleavage. As shown in fig. 30, there is no cleavage of fodrin in control or SJL/J CVE treated with UV-inactivated BeAn.

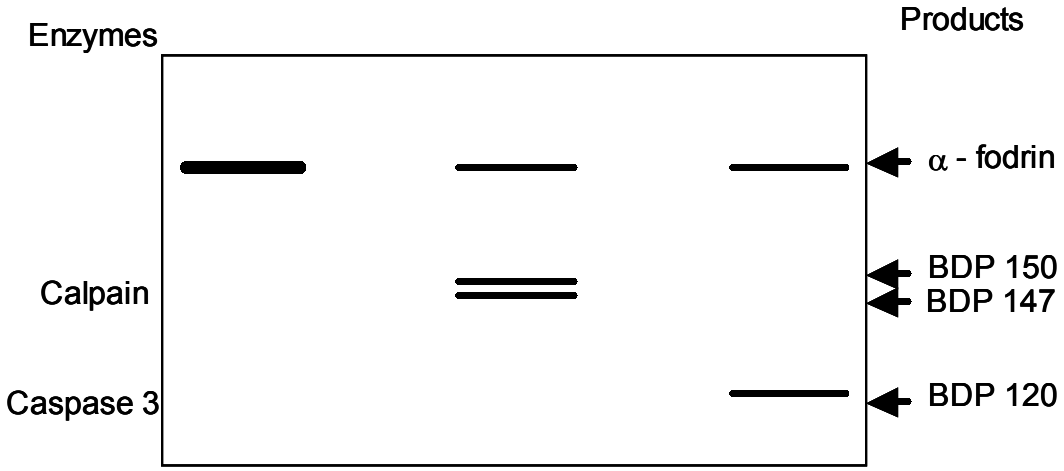

FIG. 27. Fodrin breakdown pattern. Note that both calpain and caspase can break down calpain.

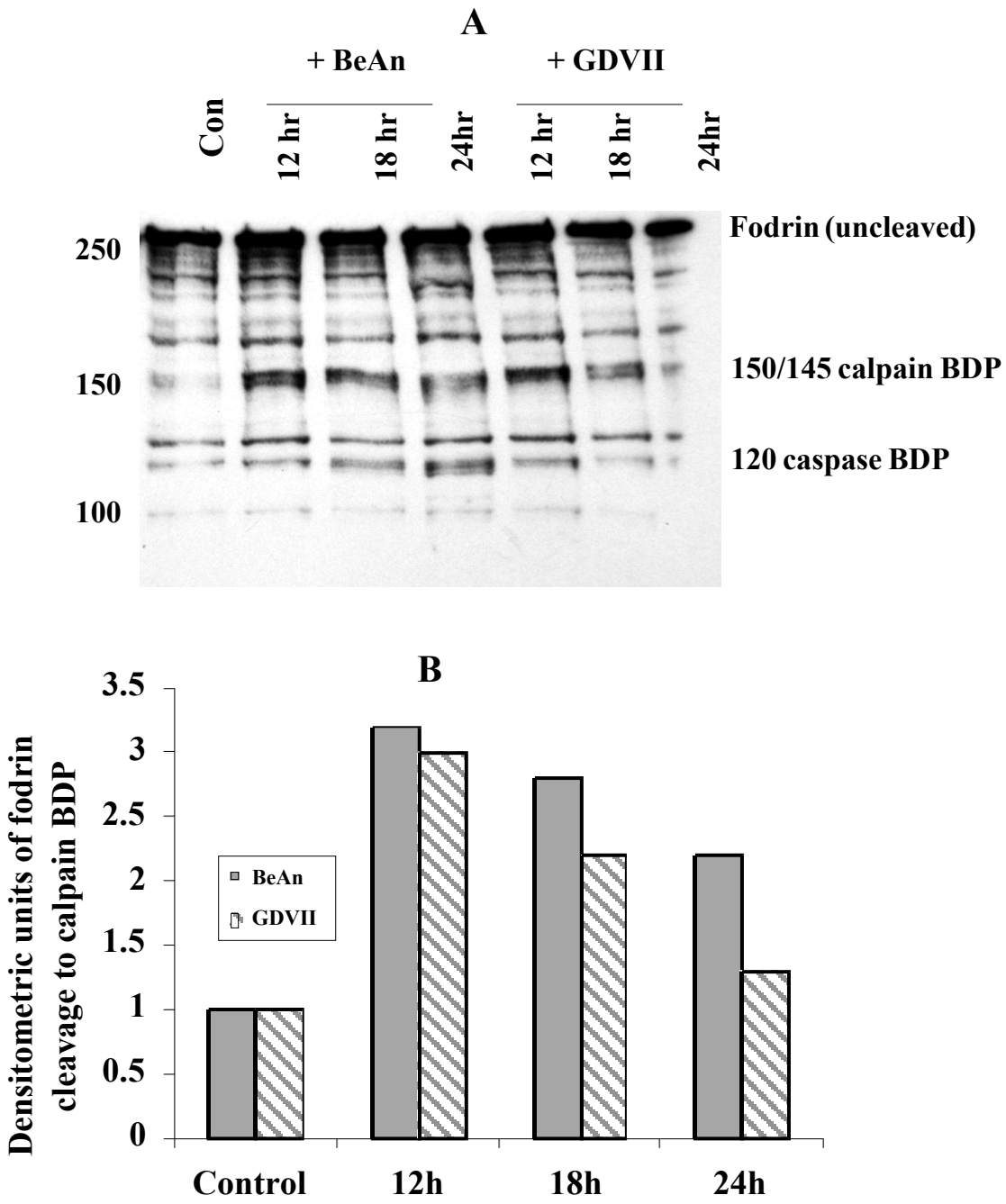

FIG. 28. Fodrin cleavage in TMEV-infected SJL/J CVE. SJL/J CVE was mock-infected or infected with BeAn and GDVII at MOI of 1 for the indicated times and immunobotted with antibody to fodrin (A). (B) indicates an increase in fodrin cleavage in TMEV-infected CVE starting at 12 h and increasing over time.

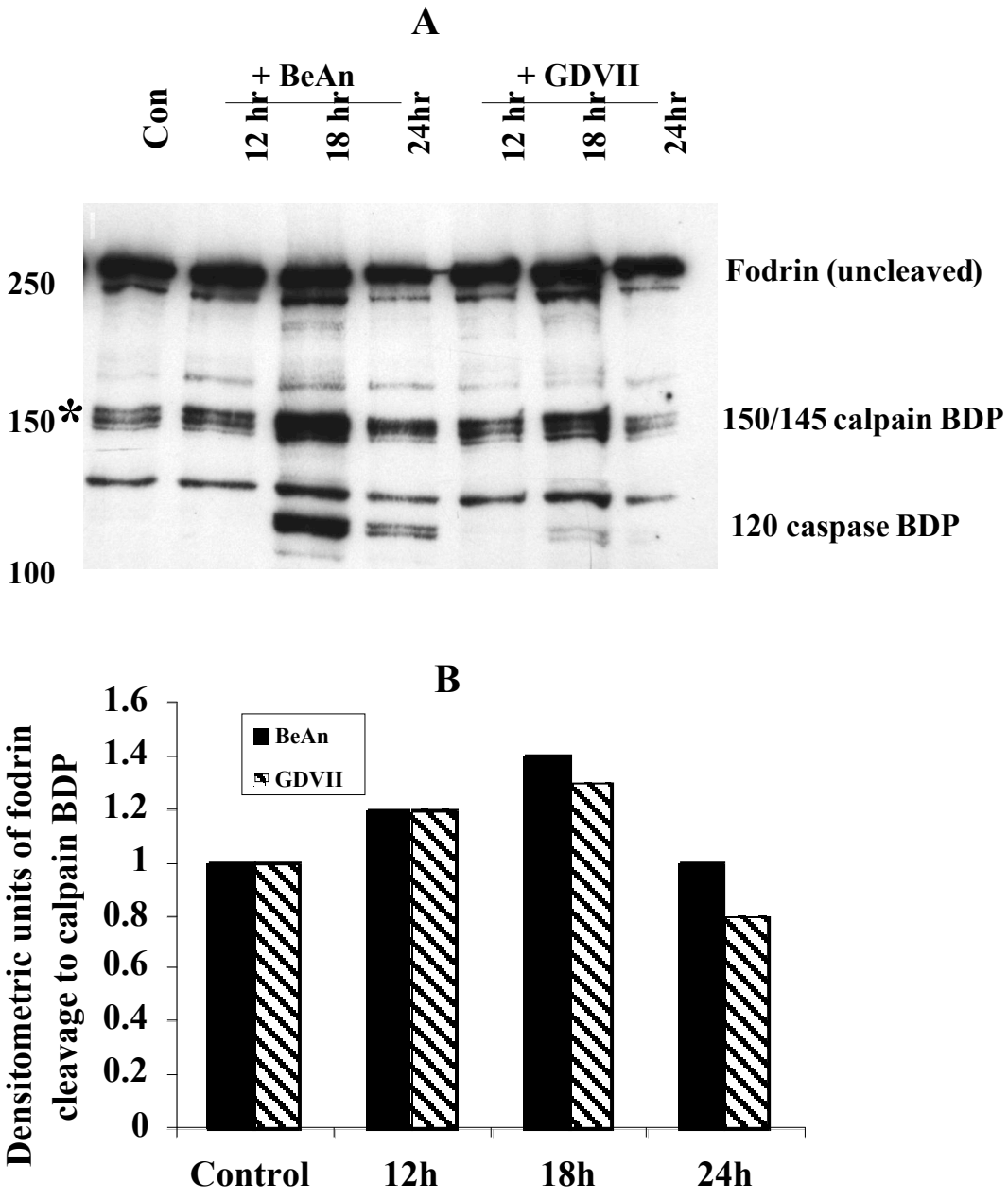

FIG. 29. Fodrin cleavage in TMEV-infected BALB/c CVE. BALB/c CVE was mock-infected or infected with BeAn and GDVII at MOI of 1 for the indicated times and immunobotted with antibody to fodrin (A). (B) indicates an increase in fodrin cleavage in TMEV-infected CVE starting at 12 h and increasing over time. (* triplet banding pattern)

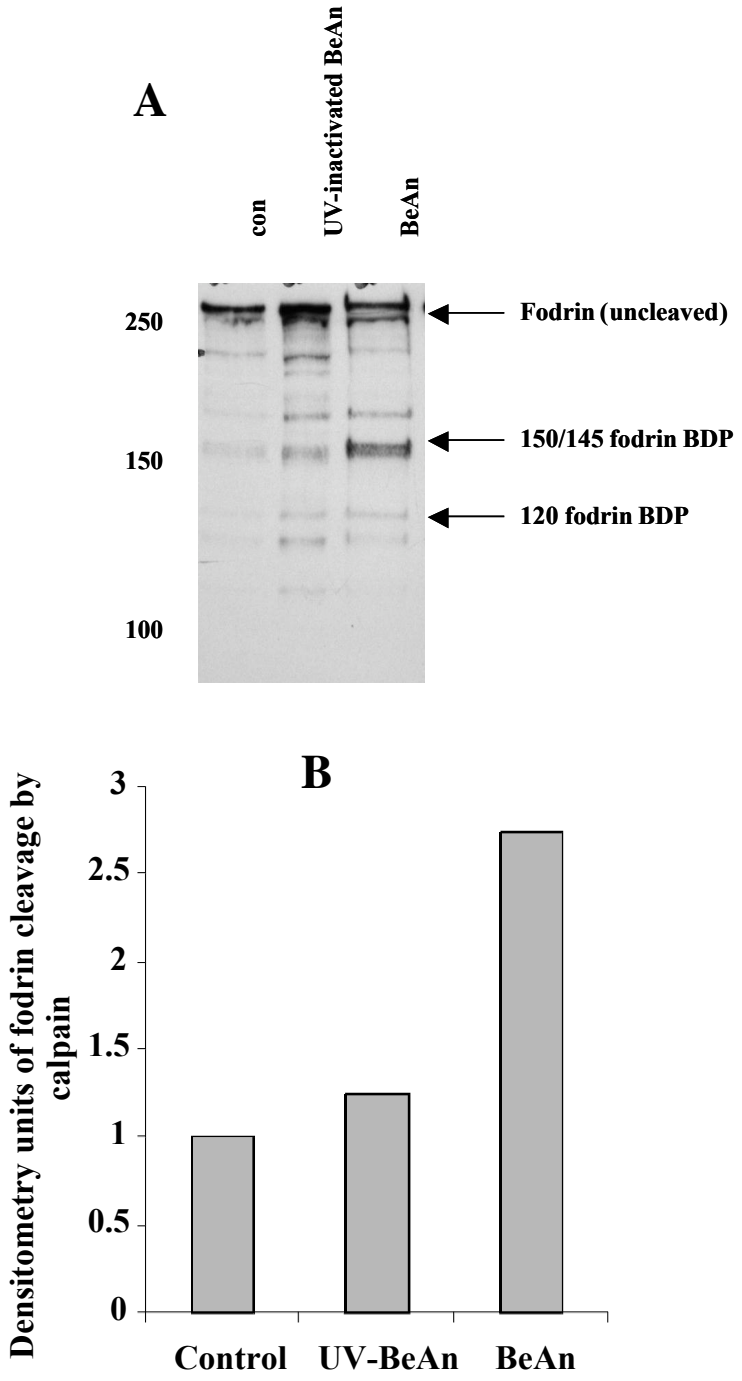

FIG. 30. Fodrin cleavage in SJL/J CVE infected with UV-inactivated BeAn. SJL/J CVE was mock-infected or infected with UV-inactivated BeAn and BeAn at MOI of 1 for 48 h and immunobotted with antibody to fodrin (A). (B) indicates densitometric analysis of fodrin cleavage. No increase in fodrin cleavage was observed in CVE infected with UV-inactivated BeAn.

DISCUSSION

Several RNA viruses including members of the *Picornaviridae* family induce apoptosis in the cell types they infect. TMEV is a Picornavirus that is known to induce apoptosis in various cell types both *in vivo* and *in vitro*. The pathogenesis of this virus is not completely understood and the route of entry of this virus into the CNS following natural infection has been pursued but not well defined. The present study, examines the pathway of induction of apoptosis in cloned mouse CVE following infection with BeAn and GDVII. Earlier work had demonstrated that following infection of SJL/J and BALB/c CVE with BeAn and GDVII, there was induction of apoptosis as measured by Annexin V, DNA fragmentation, Hoechst staining and electron microscopy. The present study shows the involvement of caspase-3 in both BeAn and GDVII infection of SJL/J and BALB/c CVE. In addition, a role for calpain - a calcium dependent papain-like neutral cysteine protease has been suggested by the findings in this report. Calpain activity appeared to be central to the apoptotic process in the CVE and it had the prominent role in the degradation of cytoskeletal elements such as fodrin as well as nuclear components such as PARP. It is still unclear at this point, whether Ca^{2+} influx caused activation of calpain or if it was a downstream effect of caspase-3 activity. However, combining results from the earlier chapter with present results, shows that caspse-3 activation began as early as 3h post-infection. The virus takes 8h to complete one round of replication and so this activation of caspase-3 may be related to host activity to prevent spread of the virus. This is, however, highly speculative and needs further experimentation. Calpain activity does not appear to begin before 18h post-

infection and may be a late effect of caspase-3 activation. Infection of CVE with UV-inactivated virus does not induce any apoptosis further strengthening the earlier argument that apoptosis is an intrinsic effect and does not involve death receptors. This was also confirmed by RPA that showed no increase over controls in the expression patterns of various molecules involved in the extrinsic pathway. It was however, interesting to note that following TMEV infection, there appeared to be a down regulation of molecules involved in the extrinsic pathway such as Fas and RIP.

Calpain has been implicated in the induction of apoptosis in neurons in case of Multiple Sclerosis (MS) (223) as well as in experimental allergic encephalitis (EAE) (221, 222, 224). Several factors can lead to the activation of calpain, which is normally held in its proenzyme form by binding of the inhibitor – calpastatin (CAST) (161). One of the ways by which calpain activation occurs is by its degradation of CAST by active caspase-3. In addition, Ca^{2+} influx can also induce activation of calpain. This occurs due to changes in plasma membrane permeability or breakdown of cytoskeletal elements. Once activated, calpain, can further the apoptotic process initiated by caspase-3 as both caspase-3 and calpain share several substrates. Some of the common substrates include PARP and cytoskeletal elements such as fodrin and spectrin. Calpain had earlier been known to mediate only necrotic death and the ability for calpain to carry out cell death through apoptosis was first demonstrated in thymocytes (230). Calpain can thus signal for both apoptosis and necrosis and unlike caspase-3 is not unique to one cell death process (248). The ability of calpain to signal for both apoptosis and necrosis may explain some of our earlier findings where the CVE infected with TMEV were initially

apoptotic, but very rapidly proceeded to secondary necrosis. Our findings do not show any increased expression of apoptotic proteins by GDVII as compared to BeAn.

Calpain related apoptotic activity has been well documented in neural cell death associated with cerebral ischemia, Alzheimer's disease (AD) (209) and Parkinson's disease (PD) (19). However, in the case of viral infections, calpain has only been implicated in reovirus infections of L929 cells (48) and neurons and in HIV infection of T-lymphocytes (215).

The role for calpain-mediated cell death in TMEV infections is very interesting because of the association with apoptosis in MS and EAE. The pathway of apoptosis induction in TMEV pathogenesis has been not clearly understood. Neurons are very sensitive to Ca^{2+} level changes and thus can undergo apoptotic cell death involving calpain. Similarly Ca^{2+} plays a very important role in the maintenance of the BBB and thus Ca^{2+} can be expected to play an important role in the induction of apoptosis in CVE, which form the BBB.

In summary, our data suggests a role for Ca^{2+} and calpain in the induction of apoptosis in CVE infected with BeAn and GDVII. There is early activation of caspase-3 but most of the effects on the nuclear components as well as cytoskeletal elements appear to be mediated in part by the activation of calpain. This needs further characterization by studies using calpain inhibitors.

CHAPTER IV

INDUCTION OF APOPTOSIS IN BRAIN MICROVESSELS FOLLOWING INTRA-PERITONEAL INFECTION WITH TMEV

INTRODUCTION

Neurotropic viruses employ a variety of methods to enter the central nervous system (CNS) from their peripheral sites. Rabies virus (45) and Herpes Simplex virus (HSV)(167) are prototypes of viruses that enter the CNS through peripheral nerves. Several other viruses such as arboviruses (51), human immunodeficiency virus (HIV) (33) and measles virus (41) enter the CNS through the hematogenous route and infection of the cerebrovascular endothelial cells (CVE). The human polyoma virus-JC virus that causes Progressive Multifocal leukoencephalopathy (PML) is believed to use B cells as a 'Trojan horse ' to traverse the blood brain barrier (BBB) (79).

Theiler's murine encephalomyelitis virus (TMEV) is a Picornavirus that enters the CNS occasionally following natural infection of the gut. CNS is a rare event resulting in a biphasic disease in susceptible strains of mice that leads to an inflammatory demyelinating disease. The route of entry of Theiler's virus into the CNS has yet to be determined. Studies suggest that TMEV enters the CNS through retrograde axonal transport (146, 198), or by replication within cells of the BBB (119, 250, 251,

259) or through infected macrophages (37, 129). There is considerable evidence pointing to the role of BBB in the transport of TMEV into the CNS.

Much of the work on TMEV has been studied following intra-cranial inoculation of TMEV. Studies by Ha Lee et al. (73), indicate that following oral infection of neonatal mice with TMEV, the route of entry of virus into the CNS most likely involves viremia and spread via the blood stream. Previous studies have indicated that CVE are permisive to TMEV infection both *in vivo* (259) and in *vitro* (251). Studies as early as 1988 by Zurbriggen and Fujinami (259) showed the involvement of CVE following intracranial (i.c) inoculation of TMEV in nude mice. Viral RNA could be demonstrated in blood vessels near the lesions as well as in unaffected regions of the brain. In addition, earlier studies by Rodriguez et al. (202) reported an increase in the major histocompatibility complex class II (Ia) antigen expression on glial cells and endothelial cells following TMEV infection intracranially.

Several other neurotropic viruses are capable of entering the CNS by replication within the CVE. The disruption of the BBB appears to be a crucial event in the development of the disease. Many of the viruses that have been shown to cause demyelination in animals also infect CVE.

In the case of HIV (33, 220) and SIV (4), in addition to replication in the CVE, these viruses have been documented to induce apoptosis in CVE. Induction of apoptosis in CVE has been demonstrated in very few instances in viral infections of the CNS. But the evidence is compelling and this could lead to a breach in the BBB and allow for inflammatory cells and more virus to gain access to the CNS and mediate damage. In

this study, we report for the first time induction of apoptosis in CVE following intra-peritoneal infection of susceptible mice (SJL/J) with TMEV. Induction of apoptosis was demonstrated by TUNEL staining and by electron microscopy.

MATERIALS AND METHODS

Virus

The BeAn 8386 strain was obtained from Dr. Howard L. Lipton (Northwestern University, Evanston, IL). The virus was grown in BHK-21 cells, and the culture supernatant containing infectious virus was aliquoted and stored at –70C before use. The viral titer was determined by plaque assay on BHK-21 cells (206).

Infection of mice

Fifteen 4 –week old SJL/J mice were infected intra-peritoneally (i.p) with $5x10^4$p.f.u. of BeAn strain of Theiler's virus in 100µl volume. The mice were sacrificed on days 1,3,7,10,14 days post infection. As a positive control, 3 mice were infected intra-cranially (i.c) (under isoflurane anesthesia) and sacrificed at day 7 post-infection. As negative control, 3 mice were injected i.p with phosphate buffered saline (PBS). At the respective time, the mice were euthanized with pentobarbitone (150mg/kg i.p) and perfused with PBS and 10% formalin intracardially. The brains were fixed in 10% formalin at room temperature overnight and embedded in paraffin. Serial sections of 5µm each were cut and stained with haemotoxylin and eosin to examine inflammation or TUNEL staining was performed to detect apoptosis.

TUNEL staining

Fragmented DNA was detected in situ by the TdT mediated dUTP nick-end labeling (TUNEL) staining method. Paraffin-embedded 5-µm-thick sections were prepared for the TUNEL method, which employed a commercially available kit (ApopTag Kit, Intergen, Norcross, GA) following the manufacturer's instructions. Briefly, deparaffinized sections were digested with proteinase K (20 mg/ml) for 15 min, and endogenous peroxidase quenched with 2% H_2O_2 for 5 min. The sections were incubated with TdT and a mixture of digoxigenin-labeled nucleotides for 60 min. This was followed by incubation with anti-digoxigenin-peroxidase for 30 min, and color development with H_2O_2–diaminobenzidine (DAB) for 3-6 min. Then, the slides were counter stained with eosin for 30 sec and cover slipped. For positive controls, specimens of mouse testes tissue were used. In these specimens, apoptosis was seen which showed typical chromatin fragmentation labeled with TUNEL. Negative controls were performed by omission of TdT enzyme from the incubation buffers.

Electron microscopy

One 4-week old SJL/J mouse was infected with BeAn i.p as previously described and sacrificed at day 15 post-infection. One mouse was used as a negative control and was injected with PBS as previously described. The brains of the sacrificed mice were collected and fixed in paraformaldehyde/gluteraldehyde fixative in 0.1 M Cacodylate buffer. The preparation of material for electron microscopy was a modification of the method by Kalt and Tandler (106). Tissues were post-fixed in 1% osmium tetraoxide,

dehydrated in a series of 10-100% ethanol and embedded in epoxy resin (Araldite 502). This was followed by polymerization in an oven for 2 subsequent steps, one at 45C for 24h and another at 60C for 24h. Ultra thin sections (70-90 nm) were prepared using a Ultrotome type 4802A ultramicrotome, stained with lead citrate uranyl acetate. The sections were examined and photographed using a Zeiss 10CA transmission electron microscope at 80kV on Kodak Electron Microscope Film 4489.

RESULTS

TUNEL staining

TUNEL-positive cells were detected in SJL/J mice infected i.p with BeAn virus (Fig. 31). Extensive apoptosis was detected in the positive controls, where BeAn was introduced i.c to the mice. No TUNEL-positive cells could be detected in PBS-treated control mice. Following i.p inoculation of the virus, TUNEL-positive cells could be detected after day- 7 post-infection. The majority of the TUNEL-positive cells appeared to be CVE cells surrounding blood vessels. The distribution and identity of TUNEL-positive cells following i.p or i.c inoculation was different. Very few TUNEL-positive CVE could be detected following i.c infection of the mice. The cells undergoing apoptosis in these samples appeared to be neurons and astrocytes. The number of CVE undergoing apoptosis following i.p inoculation of the virus increased from days 7 post-infection and by day 14 post-infection, other TUNEL-positive cells could be detected surrounding blood vessels. The blood vessels in the hippocampus had the most number of apoptotic nuclei following i.p inoculation of the virus. In case of the i.c inoculated mice, the distribution of apoptotic nuclei was more widespread.

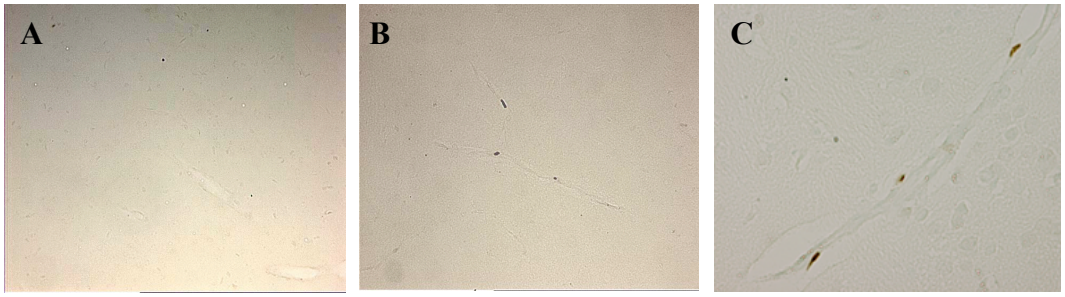

FIG. 31. TUNEL staining of brain section of SJL/J mice following BeAn infection. SJL/J mice were with either, inoculated with PBS intraperitoneally for 7 days (A), infected intraperitoneally with 10^6 pfu of BeAn for 7days (B), or infected intraperitoneally with 10^6 pfu of BeAn for 14 days (C). Note that TUNEL positive nuclei can only be observed in BeAn infected mice. TUNEL cells appear to be CVE cells. No other cell type appears to be undergoing apoptosis following ip inoculation of the virus.

Electron microscopy

Figure 32 shows evidence for apoptosis in CVE following i.p infection of TMEV at 15 days post-infection. Electron microscopic studies reveal ultra-structural details characteristic of apoptosis in CVE surrounding blood vessels in the hippocampus region. Signs of apoptosis include condensation of nucleus, loss of organelles, cell shrinkage, collapse of the lumen. There is edema surrounding these CVE undergoing apoptosis. In some instances pericytes are evident, and these have been reported to have a function in endothelial proliferation and they also express phagocytic function (237). In contrast, there are no signs of CVE undergoing apoptosis in control mice treated with PBS. There is no collapse of the lumen, or nuclear condensation and loss of organelles. In addition, no edema is observed surrounding these healthy CVE in the hippocampus.

DISCUSSION

In this study, we report for the first time, evidence for the induction of apoptosis in CVE following i.p infection of TMEV in SJL/J mice. Apoptosis in CVE was demonstrated by TUNEL staining and electron microscopy. Apoptotic nuclei were first evident at 7 days post-infection and increased in number from day 14. The distribution of apoptotic cells was different in mice infected with TMEV by i.p or i.c routes. Most of the damage in mice infected with TMEV i.p appears to be in the hippocampus region.

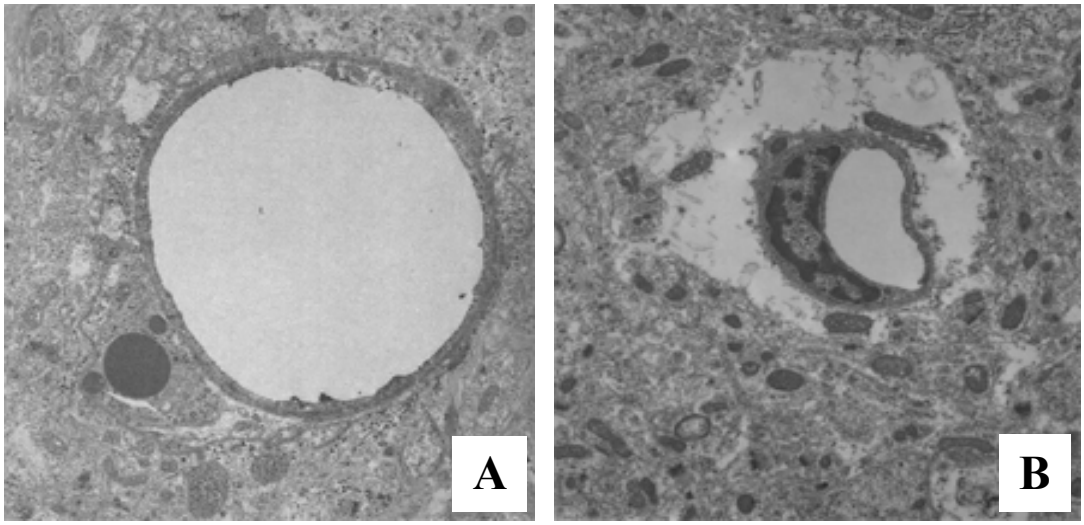

FIG. 32. Electron micrographs of brain sections of SJL/J mice following ip inoculation of BeAn. SJL/J mice were either inoculated with, PBS intraperitoneally (A) or with 10^6 pfu of BeAn intraperitoneally (B) for 15 days. Normal blood vessels are found in sham infected mice, whereas in the virus-treated mice, lumen of the blood vessels in the brain are collapsed. CVE cells lining the blood vessels show signs of apoptosis characterized by shrinkage of cells, nuclear condensation, loss of organelles. The areas surrounding the affected CVE are edematous indicating signs of loss of permeability barrier at this site.

Whereas in the i.c infected mice, the areas of the brain undergoing apoptosis appear to be more widespread. Following i.c injection of TMEV, the cells undergoing apoptosis appear to be neurons and some astrocytes. There is no evidence for apoptosis in CVE following i.c infection of TMEV.

Electron microscopic studies provide further evidence for apoptosis in CVE following i.p injection with TMEV. Induction of apoptosis in CVE is accompanied with recruitment of pericytes and extensive edema surrounding the affected blood vessels. Blood-brain barrier (BBB) is a function of the endothelial cells. They maintain homeostasis in the brain by regulating the flow of proteins and fluids into the brain. Any change in the autoregulatory process can increase CVE permeability and produce brain edema because capillaries dilate from increased luminal pressure, widening of inter-endothelial tight junctions and increase in transcapillary fluid filtration. Cells undergoing apoptosis characteristically shrink in size and this may cause widening of the inter-endothelial tight junctions and permit edema in the areas surrounding the blood vessels.

The difference in the distribution of apoptotic nuclei following i.p or i.c infection of TMEV may be related to the fact that neurotropism and distribution of a particular virus depends on the site of inoculation. For example, in case of the NSW strain of influenza virus, following i.p injection, the virus spreads by viremia (193). The viral antigens are then demonstrated in meninges, choroid plexus, ependymal cells and in perivascular locations suggesting entry through the BBB. It does not infect neurons

through this route. However, following intra-nasal inoculation of the virus, the virus spreads through nerves and viral antigens could be demonstrated in the neurons of the olfactory bulbs, trigeminal ganglion and the brain stem.

Following TMEV infection, there is evidence for involvement of CVE in the pathogenesis of the virus and CNS invasion. CVE cells were shown to be permissive to TMEV *in vitro*. Studies by Welsh et al. (251) demonstrate that BeAn replicated to similar levels in cloned CVE from both susceptible and resistant strains of mice. The levels of replication in these CVE were comparable to BHK-21 cells. In addition, persistent infection with TMEV could be established in these CVE. Persistent infection of CVE could provide a reservoir for infectious viruses, which could lead to re-infection of the CNS. This could result in characteristic perivascular infiltration of immune cells seen in TVID and cause recurrent episodes of demyelination (21). Several other viruses that have been implicated in the etiology of MS have been found to replicate in CVE. These include measles virus (MV)(41), and HSV-1 (25).

Studies by Zurbriggen and Fujinami (259) showed the involvement of CVE following i.c inoculation of TMEV in nude mice. Viral RNA could be demonstrated in blood vessels near the lesions as well as in unaffected regions of the brain. They could demonstrate viral RNA but not viral antigens in CVE suggesting either a restriction at the level of RNA replication or a difference in the sensitivities of the immuno-histochemistry reaction. This finding challenged the earlier belief of intra-axonal dissemination of the virus to the spinal cord. This also shed some light on the mode of transmission of TMEV from the gut to CNS in the natural infection.

Studies by Ha lee et al. (73) have showed that oral infection with TMEV by the per os (p.o) route, usually causes asymptomatic enteric infection in adult immuno-competent mice. Neonatal mice were more susceptible to infection through this route and showed first signs of disease 7-9 days post-infection (p.i) and this was accompanied by death 12-19 days p.i. Mice that were older than 7 days showed no signs of the disease and all survived. In the neonatal mice, the evidence pointed to the involvement of BBB in the transmission of the virus to the CNS. However, no virus was detected in the CVE from these mice and this had been attributed to the lack of sensitivity of procedures in detecting small amounts of the virus. They provide arguments against a neural spread of the virus from the gastro-intestinal tract as well as from organs such as the spleen and lymph nodes where they could be seeded following primary viremia.

Studies by Inoue et al. (84) showed that fibrin deposition in spinal cord vessels is significantly increased in i.c treated SJL/J at 30 days p.i. Batroxobin, a thrombin like defibrinogenating enzyme could reduce deposition of fibrin and also development of clinical symptoms suggesting that extravasation of fibrin could have detrimental effects. Fibrin is believed to set off a cascade that opens up the BBB and also leads to the influx of inflammatory cells into the CNS.

Earlier studies by Rodriguez et al. (201) reported an increase in the major histocompatibility complex class II (Ia) antigen expression on glial cells and endothelial cells following TMEV infection. This can be deduced as the ability of the virus to replicate in these cells and thus stimulate antigen-presenting functions. Other viruses such as Coronavirus (147) and Sindbis virus (SV) (242) can also stimulate Ia production

in astrocytes and microglial cells respectively and this may be linked to their pathogenesis. In case of SV, some endothelial cells also showed Ia antigen expression and this suggested a role for CVE in the pathogenesis of the virus. Measles virus induces Ia production on astrocytes (148).

Several viruses can infect CVE and mediate damage by causing cytopathic effects in the CVE, by up-regulation of several adhesion molecules and recruitment of inflammatory cells or by induction of apoptosis in CVE. Studies on the pathogenesis of Semliki Forest virus (SFV) have indicated the involvement of the BBB in the spread of the disease to the CNS (56, 228). SFV serves as another model to study MS. Various studies indicate that SFV is capable of infecting CVE and inducing the upregulation of several adhesion molecules such as ICAM-1 (228).

Neurovirulent simian immunodeficiency virus (SIV)(144) and human immunodeficiency virus (HIV) (33, 220) replicate productively in the CVE both *in vivo* and *in vitro*. In SIV infected monkeys, there is apoptosis of neurons, glial cells and endothelial cells. Virus replication in CVE could not only provide a mechanism for initial viral entry into the CNS, but virus-induced changes in CVE could potentially alter the integrity and function of the BBB. These virus-induced alterations could include alterations in the expression of adhesion molecules and thus promote the influx of inflammatory cells into the CNS. These alterations could also result in increased virus load in the CNS. Intranasal inoculation of avian influenza virus A can also induce apoptosis *in vivo* in vascular endothelial cells of the liver, kidney and brain (85) of the chicken. This was indicated by cerebral leakiness by detecting fibrinogen in the

extravascular space of the brain in areas with virus antigen. Viral antigens and nucleic acid could be detected in blood vessel walls *in vivo* and the virus infected CVE *in vitro*.

Viruses are normally excluded from entering the CNS in significant amounts. Introduction of several viruses through the general route, could lead to CNS involvement following a breach in the BBB by treatment with detergents, lipopolysaccharide or by mechanical injury (114, 142). Several viruses themselves can cause injury to the BBB and result in the infection of CNS through replication in the CVE (41, 228).

Thus in the present study, we provide preliminary evidence for the induction of apoptosis in CVE following i.p injection of TMEV in SJL/J mice. Induction of apoptosis in cells forming the BBB could provide a route for further entry of virus and inflammatory cells into the CNS. This study requires further characterization such as co-localization of viral antigens and TUNEL staining to indicate that induction of apoptosis in the CVE is a direct effect of the virus and not a result of soluble factors such as cytokines. Also, breakdown in the BBB needs to be demonstrated by methods such as fibrin staining.

CHAPTER V

DISCUSSION AND CONCLUSIONS

CONCLUSIONS

Relapses in Multiple Sclerosis (MS) are associated with viral infections (9, 225), but it is not known how viral infections modulate this autoimmune demyelinating disorder. Blood-brain barrier (BBB) damage caused by viral infection is one possible mechanism, since increased BBB permeability is an early and possible crucial event in the pathogenesis of new lesions in MS (109). Furthermore, some viral infections are known to cause vascular permeability changes. Vascular injury is a central feature of some hemorrhagic fevers (43) and BBB damage has been shown during human immunodeficiency virus (HIV) encephalitis (185) and uncomplicated measles virus (MV) infection (74).

The evidence for BBB damage in MS is compelling but whether this damage is mediated by virus is undetermined. As early as 1964, BBB damage had been demonstrated in autopsy of MS patients (27). Evidence such as accumulation of fibrin in perivascular areas of the brain, suggest microvessel permeability (38) and Van der Maesen et al. suggest that cerebrovascular endothelial cells (CVE) transiently express MHC II indicating immune modulatory functions (244). More recently, there has been the demonstration of abnormal tight junctions in active lesions and in normal appearing white

matter in MS patients (191) and Minagar et al. (159) have shown the increase in endothelial cells microparticles (EMP) during episodes of MS. EMPs are believed to be released following activation of endothelial cells or following apoptosis of CVE. Certain adhesion molecules such as Vitronectin receptor and β2 integrins are increased on endothelial cells in MS (227) and recently these integrins have been suggested to play a role in signaling for macrophage-mediated removal of apoptotic cells. In addition, anti-endothelial cell antibodies and circulating immune complexes have been found in MS (235). Anti-endothelial cell antibodies were elicited in another auto-immune disease – systemic lupus erythematosus (SLE) (35) and this connection between the two auto-immune diseases may be important.

It is largely unknown whether increased vascular permeability during viral infections is caused by viral replication in the CVE, virus-induced cytokines, anti-endothelial cells antibodies raised during viral infections or other yet unknown mechanisms. BBB is a function of CVE and any damage to CVE can have a profound influence on the inflammatory mileu of the brain. BBB normally inhibits viral infection of the central nervous system (CNS) and may also deter viral clearance from the CNS.

Several viruses infect endothelial cells in culture and damage them by a variety of mechanisms. Semliki forest virus (SFV)(56), HIV (33) and MV (41) enter the CNS by infection and replication within the CVE. SFV is believed to mediate damage to the BBB in addition by the expression of adhesion molecules such as ICAM-1 following replication within the CVE (228). Following dengue virus (DV) infection, antibodies against the virus cross-react with endothelial cells and mediate damage and lead to vascular leakage

(132). In the case of Japanese encephalitis virus (JEV), replication of the virus within CVE and a macrophage-derived neutrophil chemotactic factor (MDF) are believed to lead to permeability of the BBB (149). Some viruses such as HIV (220), simian immunodeficiency virus (SIV)(4), influenza A virus (85) are capable of replicating with CVE and inducing apoptosis in these cells. The effect of induction of apoptosis in CVE following infection by the virus has not been defined. Apoptosis in CVE *in vitro* has also been documented following treatment with oxyhemoglobin (172) and bilirubin (5).

Apoptosis in virus-infected cells do not greatly differ from other types of apoptosis but the viral infection may leave additional marks on the cells. Virus-infected cells undergoing apoptosis show heightened signs of apoptosis and they exhibit severe chromatin condensation around the periphery of the nucleus and active blebs that remain intact. But in some cases, the apoptosis may be so intense that insufficient numbers of cells are present to engulf the apoptotic cells bearing foreign viral antigens or the ultimate release of apoptotic bodies leading to increased inflammatory response. Induction of apoptosis itself may present as a function of the host defense against virus or may be initiated by the virus. Most DNA viruses are capable of suppressing apoptosis and carry anti-apoptotic genes as they require nuclear integrity to complete replication (116). In this case, induction of apoptosis following viral infection is a result of the host cell defense to curtail virus replication. However, most RNA viruses have relatively short replication time and have cytoplasmic sites of replication and in this case, the induction of apoptosis is beneficial to the virus and could in fact help in the release of non-lytic viruses. Apoptosis does not usually recruit an active inflammatory response. However, apoptosis

could ultimately result in secondary necrosis and the compromise of the plasma membrane releasing the virus as well as other toxic elements thus recruiting an active immune response, which may be detrimental at sites such as the brain, where post-mitotic neurons are irreplacable.

Theiler's murine encephalomyelitis virus (TMEV) is a Picornavirus that is capable of inducing a demyelinating disease similar to MS in susceptible strains of mice. Following the natural course of infection, CNS invasion, which is a rare event is achieved in a small majority of mice and can lead to a biphasic demyelinating disease -Theiler's virus-induced demyelination (TVID). The virus can be inoculated through a variety of routes such as intra-cranial (i.c), intra-peritoneal (i.p) and intra-nasal (i.n) routes. The chronic phase of the disease following i.c inoculation of the virus has been extensively studied due to its similarity at this stage to the disease pattern in MS especially with relation to the auto-immune components and inflammatory response. However, the acute phase of the disease has not been studied thoroughly.

There is considerable evidence pointing to the role of the BBB in the entry of the virus from systemic circulation to the CNS following natural infection. Following, oral route of inoculation of the virus, the entry of the virus into the CNS has been suggested via the BBB following viremia (73). CVE have been reported to be permissive to TMEV both *in vitro* (119, 251) and *in vivo* (259). Studies by Rodriguez have demonstrated an increase in MHC II antigen (Ia) on glial cells and CVE following TMEV infection i.c (202).

Like other members of *Picornaviridae*, TMEV is also capable of inducing apoptosis in a variety of cells. Members of *Picornaviridae* capable of inducing apoptosis

include coxsackie B virus (31), enchepalomyocarditis virus (EMCV) (254) and PV (44, 69, 139). PV is unique in its ability to both induce as well as inhibit apoptosis depending on the permissivity of the cell type (238). TMEV can induce apoptosis in a variety of cell types including neurons (10, 241), oligodendrocytes (241), astrocytes (180, 256), and macrophages (92-94). Whether the induction of apoptosis in these cells *in vivo* is responsible for the pathogenesis of the virus has yet to be determined. Our study adds CVE to the list of cells that are capable of undergoing apoptosis following TMEV infection. This is the first report of the only permissive cell type, apart from neurons, that undergoes apoptosis following TMEV infection. The ability of TMEV to induce apoptosis has been attributed to the permissivity of the cells to the virus in previous reports (95). TMEV-induced apoptosis has been reported as a result of virus infection since virus that is inactivated by UV-irradiation fails to induce apoptosis (95) and our studies confirms this in cloned mouse CVE. The GDVII strain is much more efficient at inducing apoptosis than the TO strains and earlier reports have demonstrated the role for an anti-apoptotic protein -L* protein in the persistence of the TO strains (63).

Our studies demonstrate for the first time the induction of apoptosis in CVE both *in vitro* and *in vivo* following i.p inoculation of the virus. This could be crucial for the pathogenesis of the virus since the induction of apoptosis in CVE could lead to damage to the BBB as seen in other viral infections and allow for inflammatory cells and more virus to enter the CNS. There appeared to be no difference in the induction of apoptosis in the CVE derived from two genetically different mice (SJL/J and BALB/c) and we could thus infer that ability to mount inflammatory response and subsequent steps may be more

crucial in the pathogenesis of the virus. GDVII induced heightened apoptosis in CVE following infection as demonstrated by several studies. However, the protein profile did not suggest an overt activation of pro-apoptotic genes following GDVII infection.

Studies reported herein, with Annexin V staining, Hoechst staining and caspase staining provide insights into the timing of induction of apoptosis, although more experimentation is required. Endothelial cells are very susceptible to the induction of apoptosis following serum deprivation due removal of growth factors present in the serum (81, 194). However, our studies indicate that CVE are not highly susceptible to apoptosis induced by 3 days of serum deprivation and this would not hinder the observation of apoptosis following TMEV infection. Like other RNA viruses, increasing the dosage of virus increased the induction of apoptosis and this appeared to be related to cytopathic effects (CPE) shown by the virus. Most viruses belonging to *Picornaviridae* have the ability to induce CPE in the cell types they infect and thus has been used as an easy marker for virus replication in cell culture following PV infection (143). CPE has been characterized by cell rounding and nuclear condensation, followed by shrinkage and detachment of cells from their support. Picornaviruses, with the possible exception of hepatitis A virus (HAV) (34, 75), induce cell alterations that culminate ultimately in cell death. The earlier finding that CPE caused by the virus could result in death of the cell is challenged by the finding that apoptosis can also lead to picornavirus-related cell death. The mechanisms of CPE and apoptosis are different. CPE consists of nuclear alterations and restructuring of cellular membrane that are involved in efficient viral replication. Apoptosis, on the other hand is a cellular suicide program and may be triggered by many

stimuli including viral infections. In picornavirus-infected organs of human or animals, cells can be found showing characteristics of either CPE or apoptosis. It is an open discussion whether the outcome of an infection depends on the balance between apoptosis and viral-mediated cell killing. Our studies indicate that increasing the titer of the virus, increased CPE as well as apoptosis and clumps of cells detached due to CPE were shown to be apoptotic with Hoechst staining.

Studies using caspase-3 and mitochondrial activation staining, indicate the activation of caspase-3 occurs as early as 3 hr in CVE infected with TMEV. This occurs earlier than nuclear condensation, which can be determined by Hoechst staining. The reduction in the apoptotic nuclei following incubation of CVE with pan-caspase inhibitors indicate that caspase-3 is possibly a very early event in the death of the cell. Whether this is a result of host defense against the virus or triggered by virus itself cannot be ascertained. The indication of mitochondrial activation following caspase-3 activation, but before condensation of the nucleus, indicates the role of mitochondria in the cascade of events. The involvement of mitochondria related proteins such as bax, bcl-2, bak, bid and cytochrome-c needs to be determined. This could be crucial to our studies as the involvement of Ca^{2+} in our system is definite.

Consistent with other findings, inactivation of the virus following UV-irradiation did not induce apoptosis in CVE thus enforcing the fact that live virus was required and apoptosis was a result of intrinsic factors. Several viruses such as hepatitis B virus (77) and SIV (98) can induce apoptosis by the recruitment of Fas and other surface molecules. This does not appear to be the case with TMEV. Interestingly, in case of TMEV infection

of CVE, there appeared to be a down regulation of surface molecules involved in apoptosis such as Fas and RIP. HIV, which is known to be a strong inducer of apoptosis in several cell types is believed to down regulate Fas in lymph nodes of patients with HIV(249). The importance of this finding is not determined. Down regulation of Fas has mostly been attributed to the ability of the virus to inhibit apoptosis and this does not appear to be the case in CVE infected with TMEV.

Electron microscopic studies on CVE infected with TMEV indicate that following infection, the CVE showed signs of apoptosis clearly indicated by signs such as cell shrinkage, surface blebbing, nuclear condensation and loss of organelles, but the relative preservation of the cell membrane. But by 24h following infection, the cells appeared to rapidly show signs of secondary necrosis. Classically, apoptotic cells package into apoptotic bodies, which are engulfed by neighboring cells or by macrophages, but occasionally, cells are known to progress into secondary necrosis following viral infections. This finding by electron microscopy is supported by Annexin V data, which shows passage of cells into secondary necrosis by display of phosphotidyl-serine early in apoptosis and staining with propidium-iodide in addition later indicating cell membrane compromise. This may also be explained by our finding later that calpain is involved at a later stage in the apoptotic process following TMEV infection of CVE.

Calpain is known is to trigger for apoptosis as well as necrosis and has been a strong candidate for studies in apoptosis in MS as well as experimental allergic encephalomyelitis. We propose for the first time the involvement of calpain in TMEV infection. Calpain activation is central to the apoptotic process in neurons and increased

calpain activity was observed in reactive astrocytes, activated T cells, and activated mononuclear phagocytes in and adjacent to demyelinating lesions (223) in MS but has never been demonstrated in CVE following viral infections. The involvement of calpain in the induction of apoptosis could be crucial as this could lead to loss of membrane integrity and thus could amplify the inflammatory response at the site of infection instead of curtailing inflammation due to apoptosis.

In vivo studies following i.p inoculation of TMEV has been attempted very infrequently. Considering the enteric source of infection, this route of infection would be ideal to study a more naturalistic model of infection and route of entry of virus into the CNS. Our studies with i.p inoculation of the virus indicate the alterations in CVE of the hippocampus area. CVE in this area appeared to be undergoing apoptosis and large areas of edema were evident surrounding blood vessels indicating increased permeability. The lumen of the blood vessels appeared collapsed and the CVE appeared to be shrunken but the cell membrane appeared intact.

Thus with the evidence provided by the studies, we propose the following theory. Following natural enteric infection of mice, TMEV enters the CNS following general viremia and primary seeding in other organs such as spleen and liver and lymphoid tissue. At the BBB, TMEV enters and replicates in CVE, and induces CPE as well as apoptosis in them. The two processes might be mutually dependent or totally exclusive events and this cannot be determined at this moment. Activation of caspase-3 is central to the apoptotic process, but unlike most processes, caspase-3 activation is followed by calpain activation and this leads to nuclear breakdown events as well as cytoskeletal

changes. Induction of apoptosis in areas such as the BBB in the brain could have a variety of effects.

One of the hallmarks of apoptosis is cell shrinkage and considering the characteristics of the CVE that form the BBB, this could be crucial. Other reports such as osmotic shrinkage in a hypertonic environment in the BBB could result in cell shrinkage and widening of the tight junctions (165, 210). These changes can cause increase in CVE permeability and produce brain edema as capillaries dilate from increased luminal pressure. Similar effects due to apoptosis of CVE can be hypothesized.

Unlike necrosis, apoptosis leads to cell death with minimal inflammatory response. Our finding of the involvement of calpain in the apoptotic process in CVE might result in the compromise of the cell membrane and thus release toxic cell components into general circulation thus eliciting a huge inflammatory response. Apoptotic cells display phosphotidyl-serine on their surface, thus recruiting macrophages to the site, but involvement of calpain may mediate a much higher inflammatory response. Studies with human umbilical vein endothelial cells (HUVEC) have shown that endothelial cells induced to become apoptotic by oxidative damage, result in the recruitment of complement (160). CVE undergoing apoptosis could also cause similar changes and result in inflammation at the site. Thus, unlike common belief, apoptosis of cells can also cause increased inflammation like necrosis.

FUTURE DIRECTIONS

The present study provides preliminary evidence for the involvement of apoptosis of CVE in the pathogenesis of TMEV. This model needs to be strengthened by the following studies:

1. Determination of virus load in various organs following i.p inoculation over time. This will increase our understanding of TMEV invasion of the CNS. Some of the proposed organs to be studied include spleen, heart, and liver. It is believed that TMEV infection naturalistically involves a viremic stage. Viral load in the blood at various times following i.p infection is proposed to understand this viremic stage better.
2. Determination of virus distribution in the brain following i.p inoculation and co-localization studies for virus and apoptosis. Employing methods such as fibrin deposition should be undertaken to determine extent of blood-brain barrier damage.
3. Determination of genes involved in apoptosis at the BBB and the use of calpain and caspase inhibitors to prevent the apoptotic effects of the virus.

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