Autoantibodies to Cerebrovascular Endothelial Cells in Virus-Induced Demyelination

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Abstract

The elucidation of the pathogenesis of multiple sclerosis (MS) has been an ongoing process for many years. MS is a relapsing disease with demyelination of the central nervous system's (CNS) white mater; symptoms are motor weakness, impaired eyesight, diplopia, spasticity, lack of sensation, and many other neurological manifestations. Scientists employ animal models to study the demyelinating process, and the two most widely used animal models simulating MS are experimental allergic encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus (TMEV). Our research focused on TMEV and its pathogenesis. We performed intracerebral infection of TMEV in CBA mice. The demyelination of the CNS brought about spasticity and paralysis of the hind limbs. Many have challenged and questioned the notion whether MS and Theiler's murine virus disease (TVID) are autoimmune diseases. There have been reports of autoantibodies against the endothelial cells (Tanaka et al, 1987) and myelin basic protein (MBP) (Cash et al., 1992) in the sera of MS patients and mouse myelin (Welsh et al., 1987) and MBP (Rauch et al., 1987) in the sera of TMEV infected mice. Enzyme linked immunoabsorbant assay (ELISA) was used to determine if the pathogenesis of this virus involved the production of autoantibodies to cerebrovascular cells (CVE). We made sera dilutions of 1:200 and 1:400 and performed ELISA's on the sera of numerous mice. There were significantly higher amounts of antibody binding (95% confidence) in infected and sick sera as compared to the control sera. This indicated the presence of autoantibodies to CVE in Theiler's infected mice. These results encourage the thought that autoimmunity is involved in MS and TVID either as a primary or secondary phenomenon. Such information may some day provide an answer for treating or preventing MS and other demyelinating diseases.

Introduction

Max Theiler first described Theiler's murine encephalomyelitis virus (TMEV) in 1934 when he observed that this virus affected the central nervous system in mice. The mice exhibited paralysis of the posterior limbs and was originally used as a model for poliomyelitis (Theiler, 1934). Since that time over twenty TMEV strains have been identified with some causing demyelination. Scientists and researchers have placed a great deal of effort into understanding the virus. TMEV is a naturally occurring enteric pathogen of mice which infects the gastrointestinal tract and travels to the central nervous system via bloodstream or axonal transport. This virus produces a biphasic disease demonstrated with acute encephalitis followed by chronic demyelination (Lipton, 1975).

Many consider that the TMEV is the best animal model relating to multiple sclerosis (MS). TMEV infection elicits clinical signs and pathology reminiscent of those seen in MS. In addition, there does not seem to be any direct correlation between the severity of demyelination and viral titer in the tissue (Waksman, 1985). The mouse infected with TMEV displays autoimmune characteristics also seen in MS, possibly involving autoreactive T cells, autoantibodies, or both (Welsh et al., 1990).

TMEV belongs to the *Picornavirus* genus, and genetic characterization suggests that it resembles *Cardiovirus.* The different strains of the virus are categorized into two main groups. One category, the GDVII subgroup, consists of the most virulent stains of TMEV, GDVII and FA, which are usually fatal upon early onset . In nature, TMEV rarely causes any serious problems. However, Liu et al (1967) illustrated that experimental administration of GDVII intracranially, intranasally, or orally eventually brings about death. The remaining strains fall into the less virulent group, the TO subgroup, demonstrating acute poliomyelitis and persistent demyelination of the central nervous system (Lipton et al., 1975). A great deal of interest focuses on the less virulent strains of TMEV because of their similarities with

MS. Following intracranial infection with BeAn, 20% of CBA strain mice displayed acute poliomyelitis, during the first month of disease. In the late phase of the disease, 70% of mice develop spastic paralysis. Viral persistence in the central nervous system (CNS) of susceptible strains of mice leads to chronic inflammation (Lipton et al., 1978) and the demyelination of white matter in the CNS (Theiler, 1937).

Evidence points towards an immunological role in the persistence of TMEV and the effective destruction of the CNS. The major histocompatibility complex H-2 genes contributes to the susceptibility of the TMEV infection and demyelination. (Rodriquez et al., 1986) Melvold et al. (1990) state that risk factors involved in TMEV demyelination are the genes for major histocompatibility complex (MHC) and the genes encoding the ß chain of the T-cell receptor. Analyzing the susceptible DBA/2 and resistant C57BL/6 strains, Mevold et al. discovered that the H-2D locus and a non-H-2 gene located on the centromeric end of chromosome 3 play important roles. Experiments demonstrate that MHC class II may contribute to the demyelinating process. Interferon gamma's ability to induce MHC II expression on astrocytes (Borrow., 1989 and Borrow et al., 1992) and cerebrovascular endothelial cells (Welsh et al.) directly related to the susceptibility of demyelination. Researchers witness aberrant Class II expression in astrocytes of TMEV infected animals (Rodriguez et al., 1987); Friedmann et al. (1987) alleviated the severity of the disease using monoclonal antibodies targeted against MHC Class II.

Many theories hypothesize the reasons why TMEV persists in the CNS. Persistence may arise through antigenic variation of the viral polypeptide (Scott et al., 1979), viral-membrane association (Frankel et al., 1987), destruction of the TMEV antibody neutralization site at the carboxyl end of VP1 (Roos et al., 1989), and restricted viral replication (Cash et al., 1985). In addition, the exact mechanism by which demyelination occurs is not known. Nevertheless, several models have been proposed to explain this event. Demyelination may occur because of viral lysis of

oligodendrocytes, the myelin producing cells of the CNS. Rosenthal et al. (1986) successfully induced demyelination by infecting the nude mice. Virus replication was evident in oligodendrocytes (See Figure 1a) and cytotoxic T cell damage to the virus infected oligodendrocytes are witnessed (See Figure 1d). Yet, oligodendrocytes are not major players in the demyelination process during the initial stages of the disease in immunocompetent mice since these cells do not present viral antigens initially.(Dal Canto et al., 1987) Secondly, the immune system seems to produce an autoimmune response toward the myelin, closely resembling the signs of experimental allergic encephalomyelitis (EAE) (See Figure 1b). Lastly, 'bystander' mechanism may lead to demyelination (See Figure 1c). The persistent infection causes the release of cytokines and proteases by delayed type hypersensitivity (DTH) T cells and macrophages, respectively. Delayed type hypersensitivity is an immune-mediated inflammatory reaction occurring 24 to 48 hours after an antigenic challenge, characterized by vasodilatation and cellular infiltration. DTH T cells in the CNS recruit macrophages which mediate the degradation of the myelin sheath. Aberrant MHC Class II expression on astrocytes results in increased antigen presentation thus eliciting a stronger T cell response (Rodriguez et al. 1986) and increasing inflammation (See Figure 1e). It appears that humoral immunity plays the major role in1 viral clearance while cell mediated immunity also aids in viral clearance, albeit minor (Welsh et al., 1987).

The blood-brain barrier (BBB) immunologically protects the CNS. The BBB consist of cerebrovascular endothelial cell with tight junctions and the end feet of type 1 astrocytes (Janzer et al., 1987). Furthermore, the CVE's limited amount of pinocytic vesicles and characteristically high resistance (1900/cm^2) add to the limited permeability of the BBB (Joo et al., 1989). Studies demonstrate that there is very low MHC expression in the central nervous system thereby hindering the antigen presenting capabilities of these cells (Suckling et al., 1986). Despite the fact that very

small ions such as lanthanum did not permeate the BBB (Bundgaard, 1982), access into the central nervous system is still possible. Regular T cell movement occurs across the blood brain barrier even in normal animals although lymphocyte trafficking is somewhat limited (Wekerle et al., 1986). Patients with progressive multiple sclerosis displayed more rapid T cell trafficking (Hafler et al., 1987). It should also be noted that the CNS possess leaky areas where the passage of molecules and cell is easier. These include the hypothalamus, area postrema, and spinal and cranial roots.

A great deal of interest has evolved around TMEV's access into the CNS. Theiler's virus travels to the CNS, an immunologically protected system, via axonal transport or the blood stream and enters the CNS through the blood brain barrier (BBB). The virus directly or indirectly interacts with cerebrovascular endothelial cells (CVE) which forms the BBB and penetrates into the CNS. Furthermore, immune cells, such as macrophages and T cells, also gain access into the system and mediate demyelination.

Theiler's virus has been shown to infect the CVE in vitro and may also occur in vivo which would adversely affect the BBB. CVE obtained from strains of mice that are susceptible to Theiler's virus-induced demyelination express MHC Class II when stimulated with IFN-gamma; the resistant strains of mice did not (Welsh et al.). The CVE cells of susceptible strains may be capable of presenting viral antigens and myelin basic proteins to T cells (McCarron et al., 1985). Also astrocytes from susceptible strains of mice, functioning as antigen presenting cells by expressing MHC Class II, act as accessory cells for T cell and B cell activation by releasing IL-1 (Fontana et al, 1985). After viral infection and antigen presentation, lymphocytes encounter the viral and CNS antigens, release lymphokines, and further stimulate the immune system by "calling" other lymphocytes.

The disruption of the BBB observed in patients with multiple sclerosis may be caused by immune mediated reactions directed against CVE cells or smooth muscle

cells that compose the BBB. MS patients with disrupted BBB had increased vascular permeability, more pinocytic vesicles in cerebral venules, and a large number of plaques located at the BBB. The detection of autoantibodies to CVE has been reported in MS patients (Tanaka et al., 1987). Furthermore, injected CVE cell membranes induced autoimmune encephalomyelitis in rhesus monkeys . The monkeys presented chronic neurological illness, demyelination and remyelination, and localization of mononuclear cells around the blood vessels of the central nervous system (Tsukada et al., 1988). Thus it is feasible that antibodies against CVE play a pathogenic role in inflammatory demyelinating diseases such as MS, EAE, and Theiler's virus-induced demyelination. Welsh et al previously documented the presence of autoimmune reactivity in Theiler's virus-infected mice. These animals have serum anti-myelin antibodies and T cell responses to myelin. In the current proposal, we intend to investigate whether Theiler's-infected mice produces autoimmune responses to components of the blood-brain barrier (BBB).

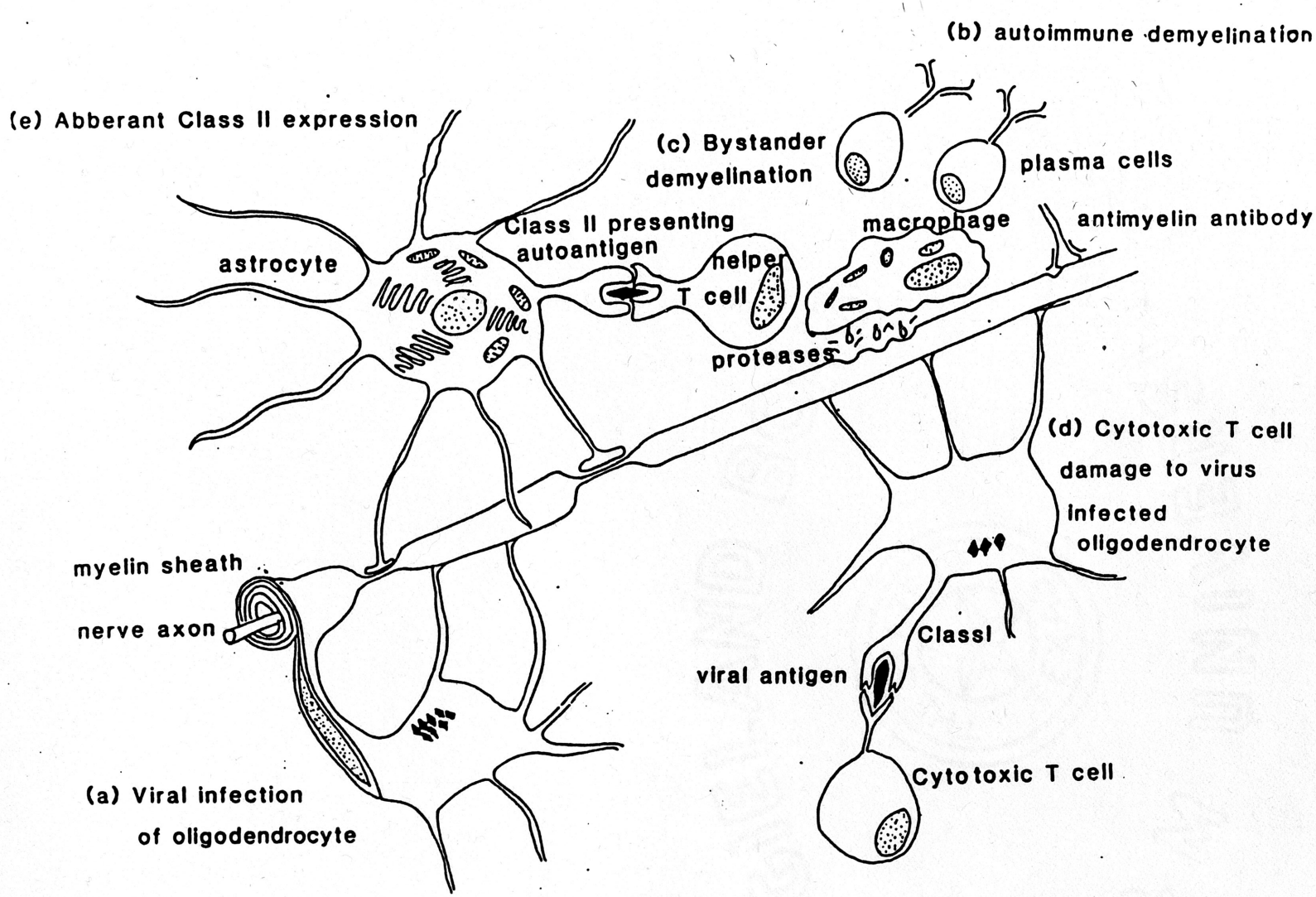

Figure 1: Diagram illustrating the some of the mechanisms that may be involved in the initiation of demyelination induced by Theiler's virus

Welsh, C., Blakemore, W., Tonks, P., Borrow, P., and Nash, A. (1989) Theiler's murine encephalomyelitis virus infection in mice: a persistent viral infection of the central nervous system which induces demyelination. In: dimmock N, ed. Immune Responses. Virus Infections and Disease. Oxford University Press. pp. 125-147.

Materials and Methods

Infection of mice

Twenty CBA and SJL 5 week old female mice (obtained from Harlan Labs) were infected intracerebrally with 10^4 of the DA strain of Theiler's virus; the control mice were infected intracerebrally with phosphate buffer solution (PBS). The mice were monitored weekly for clinical signs of demyelination. The mice were then bleed periodically from the tail vein, and the sera were stored at -20ºC. See Figure 2. The mice were scored on a scale from one to five. The two main categories were infected, healthy mice and infected, sick mice. Infected, healthy mice displayed no signs of demyelination and appeared normal while infected, sick mice exhibited spasticity of the hind limbs, weight loss, and incontinence.

Culture of cerebrovascular endothelial cells

Dr. Welsh's lab has established non-transformed mouse CVE lines from BALB/c, CBA, and SJL/J mice.(See Figure 2) The cells were cultured in endothelial cell growth media (EGM) which consists of IMEM supplemented with 10% FCS, 70 µg/ml Endothelial Cell Growth Factor (collaborative Research), 40 µg/ml heparin (sigma), 2mM glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin. Passaging of CVE was achieved by trypsinization every 3-4 days.

Assay for antibodies against CVE using ELISA (Enzyme Linked Immunoabsorbant Assay)

The cultured cells were trypsinized, resuspended, and subsequently plated on a 96 well plate. The cells were incubated at 37ºC for 3 days in order for the CVE cells to form a monolayer. The cells were then washed twice with PBS containing Ca^+ and Mg^+ and fixed with ethanol and acetate

(1:1). The fixation process takes thirty minutes at 4ºC. After fixation the cells were washed twice in PBS and were blocked by adding 5% FBS in PBS for 30 minutes at 24ºC in order to block the unbound sites. Antibodies bind non-specifically to the plastic and the cells do not completely cover the wells.

Serum obtained from the virus-infected mouse was diluted to1:200 and 1:400 in a solution containing 5% fetal bovine serum (FBS) in PBS. One hundred milligrams of the dilution was added to wells and incubated at 37ºC for 1 hour. Afterward, the cells were washed once again in PBS, and peroxidase labeled goat anti-mouse antibody was added. This antibody was placed in a dilution of 1:200 with 5% FBS in PBS. The goat anti-mouse antibody binds to the Fc receptor of any bound anti-endothelial antibodies. The incubation time was one hour at 37ºC. The plates were then washed three times in phosphate buffered saline containing 0.05% Tween 20 (PBS+Tween). The substrate was added and incubated for 30 minutes in the dark. The substrate contains 20 ml of citrate-phosphate buffer at pH 5.0, 0.01 g of OPD (o-phenylenediamine dihydrochloride), and 5.33 µl of hydrogen peroxide. Preparation of the citrate-phosphate buffer require 25.7 ml of 0.2M dibasic sodium phosphate, 24.3 ml of 0.1M citric acid, and 50 ml of deionized water. The substrate attaches to the peroxidase and produces an orange-brown end product. The reaction was terminated with 3N HCL, and the optical density read at 490nm. See Figure 3.

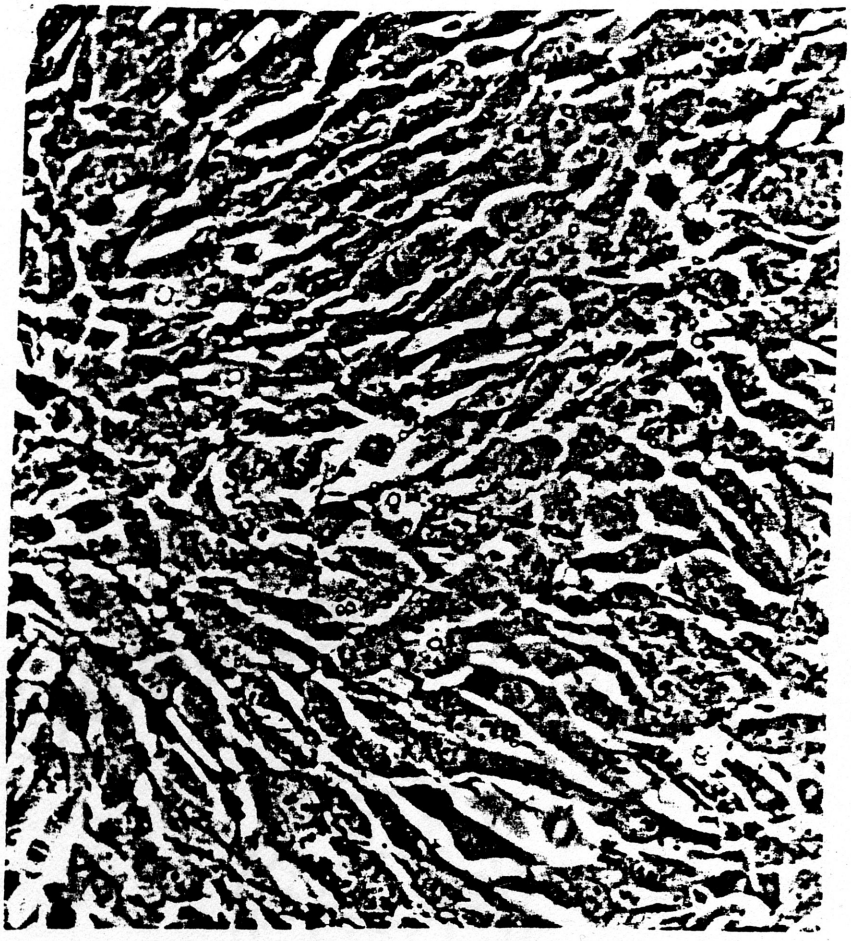

Figure 2: Monolayer of murine cerebrovascular endothelial cells

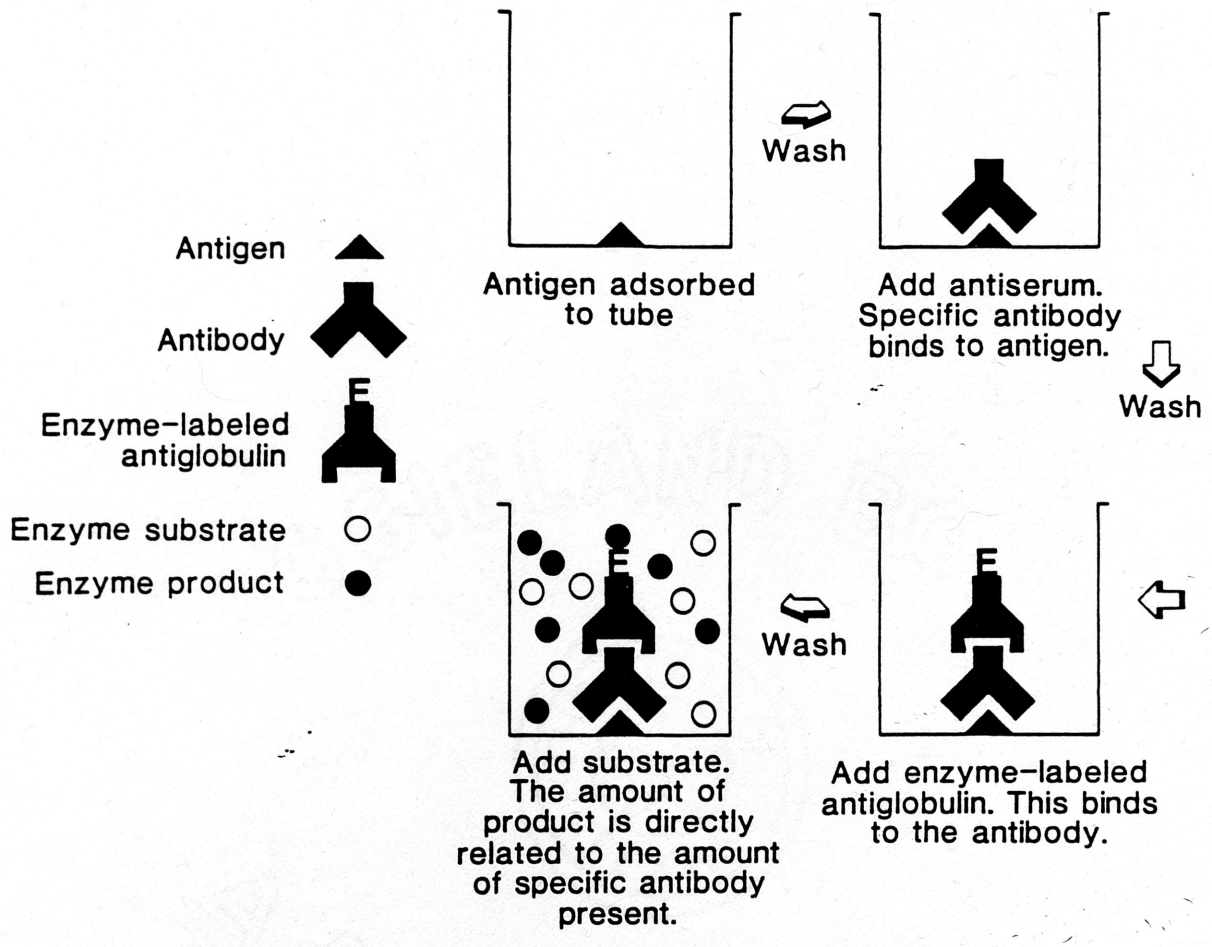

Figure 3: Enzyme-Linked Immunosorbent Assays

Tizard, I. (1987) Veterinary Immunology. W.B. Saunders Company.Philadephia, p. 134.

Statistical Analysis

We used the small sample t test in order to determine if there was a significant difference between the control and the test. The summary of the paired t test is as follows:

Null Hypothesis: $H_o: u_1-u_2 = 0$

Test Statistic:

$$t = \frac{X_1 - X_2}{\frac{S_p^2}{N_1} + \frac{S_p^2}{N_2}}$$

Alternative Hypothesis: $x_1 - x_2 <> 0$

where rejection region is either t> t critical value or t<t critical value. T critical value is determined based on N_1+N_2-2 degree of freedom.

Note: 1) N_1 is the sample size of set #1 (control)

N_2 is the sample size of set #2 (test).

2) u_1 is the mean value of population #1.

u_2 is the mean value of population #2

3) x_1 is the mean value of population #1.

x_2 is the mean value of population #2

4) s_1 is the variance of population #1.

s_2 is the variance of population #2

5) $$S_p^2 = \frac{N_1-1}{N_1+N_2-2} S_1^2 + \frac{N_2-1}{N_1+N_2-2} S_2^2$$

S_p^2 is the estimation of the common variance.

Results

Serum from 20 Theiler's virus infected mice and 5 controls were tested for binding to CVE cells. Three ELISA wells were used for each specific serum dilution. The optical densities were read and averaged. We applied the small sample T test in order to determine if there existed any significance among the three categories of mice, control, infected, and sick. The statistical analysis demonstrated that there was significant differences with 95% confidence between control and infected mice and between control and sick mice at 1:200 (Figure 4) and 1:400 dilutions (Figure 5). Furthermore, a confidence level of 99.9% was seen in the 1:200 dilution of control versus infected mice and in the 1:400 dilution of control versus sick mice. These results indicate that there was development of autoantibodies to the cerebrovascular endothelial cells of infected and sick mice. In contrast, the sera of non-infected control mice did not produce any increased levels of antibody binding, despite the fact that they had received an intracerebral inoculation which would damage the BBB.

The development of autoantibodies to CVE was followed in a time course study in a number of animals. Figure 6 demonstrates the development of antibodies in a Theiler's infected mouse at one, two, and three months post infection compared to a control animal. Peak titers appeared at one month post infection when the mouse was clinically healthy. By six weeks post infection, the animals suffered from signs of demyelination, and at eight weeks the level of autoantibody had decreased to pre-bleed levels.

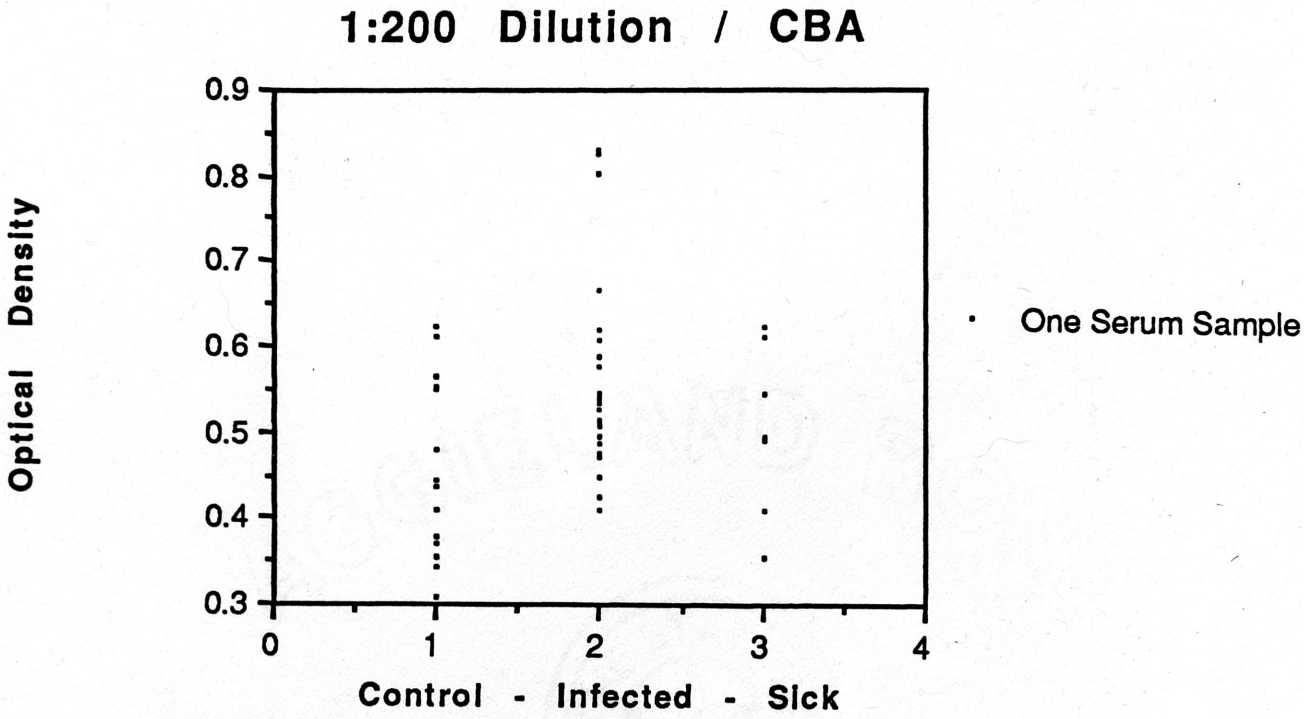

Figure 4: The optical density at 490 nm of 1:200 CBA mouse serum during different phases.

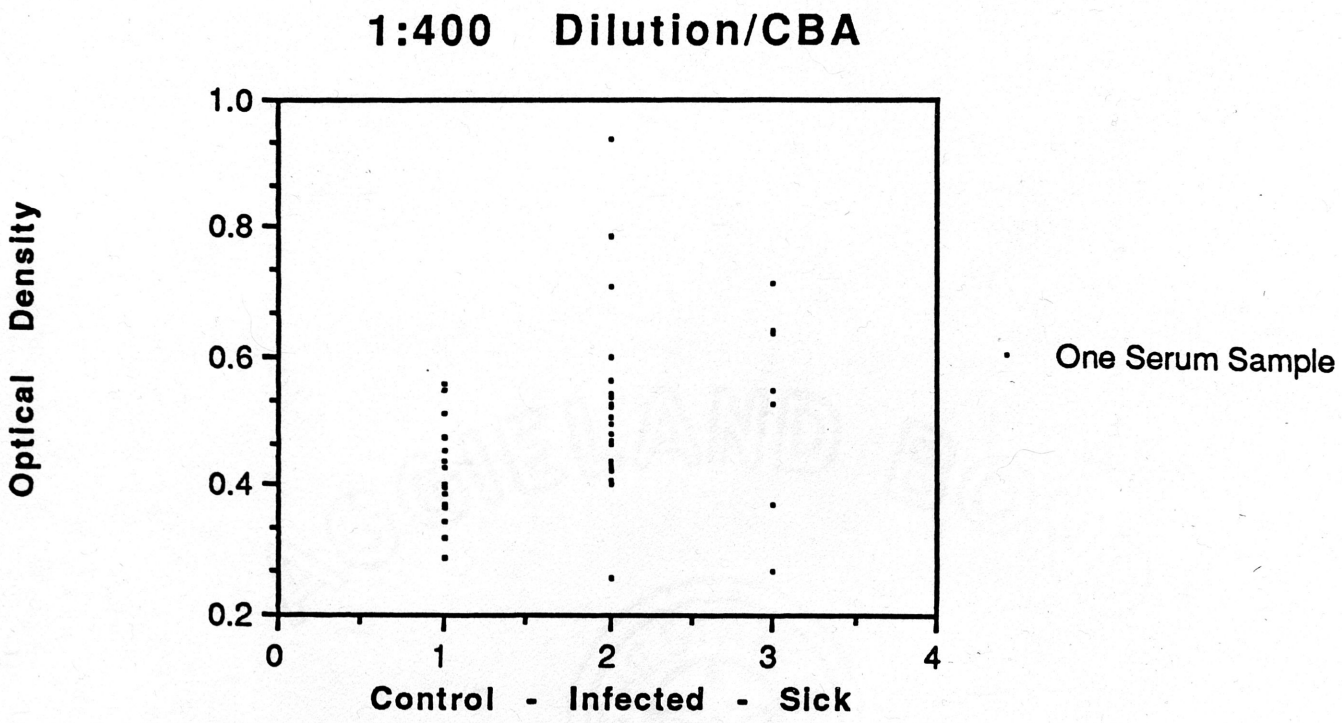

Figure 5: The optical density at 490 nm of 1:400 CBA mouse serum during different phases.

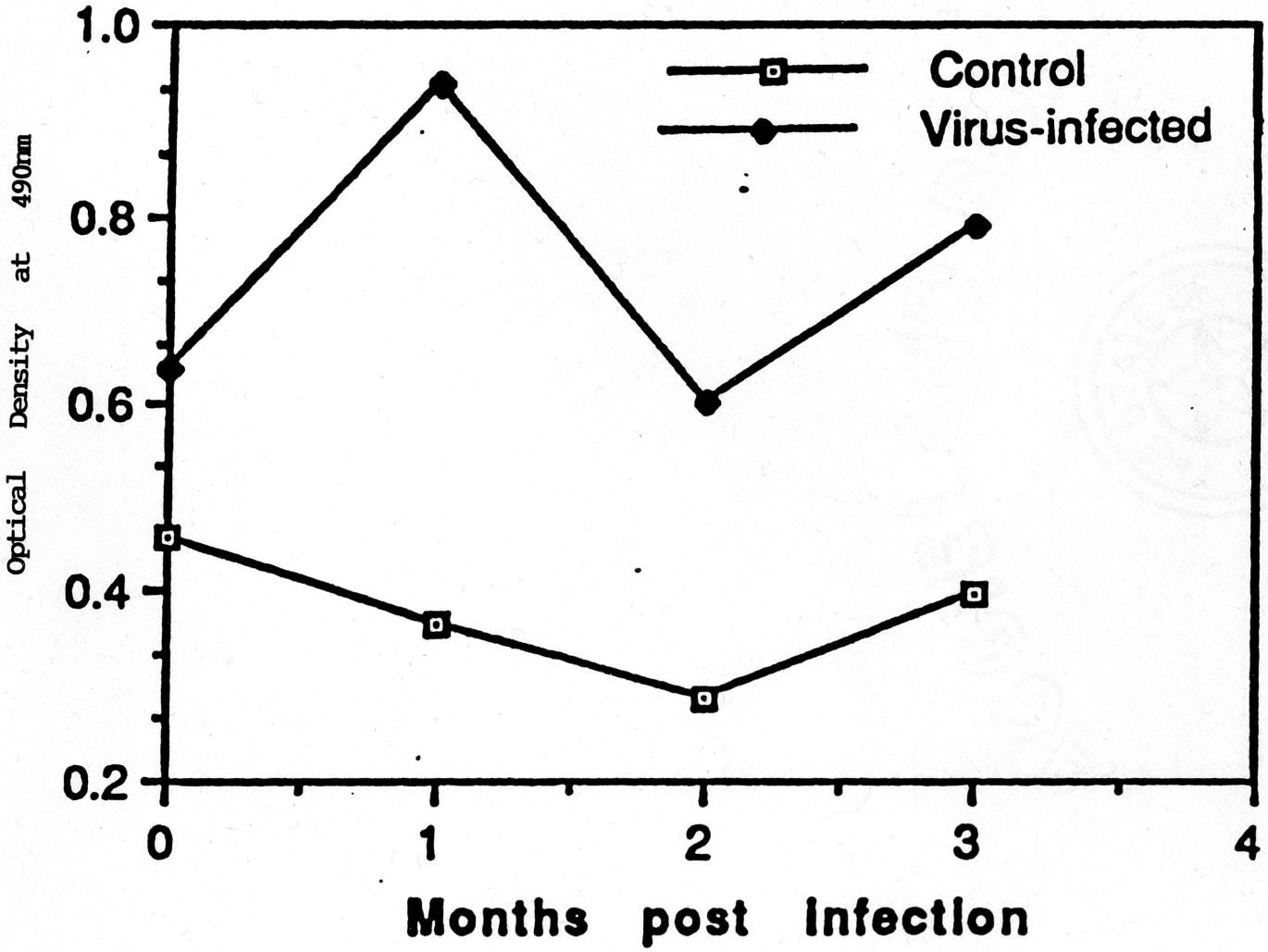

Figure 6 : The optical densities at 490 nm of a control and virus infected mouse serum at different times post infection

Discussion

Our data supports the hypothesis that during TVID there exists an autoimmune event toward the self antigens of the CVE cells. It has already been documented that the Theiler's virus brings about serum antibodies to myelin (Welsh et al., 1987) and myelin basic protein (Rauch et al., 1987). Furthermore, autoantibodies have been detected against CVE cells (Tanaka et al., 1987) and MBP (Cash et al., 1992) with MS.

According to our statistical analysis of the optical densities (OD) of the ELISA's, the serum of the infected and sick mice binded to a significantly greater number of antigens on the CVE cells than the serum of the control. This indicates that during the stages of post-infection there was evidence of autoantibodies reacting to the autoantigens found on the surface of the CVE cells. Damage to the BBB and the CNS exacerbates the situation triggering the immune system even more since the immune system has entered into immunologically "protected" territory. At this stage of the disease, the autoantigens found in the CNS may be altered eliciting an immune response. Ethanol acetone, the solution used to fix the cells, permeates cells allowing access of the antibody to the autoantigen which may be an internal or external membrane component. Serum taken from the sick mice does not contain the levels of antibodies seen in healthy infected mice. This may be due to the immunosuppressive effects of the virus in extremely ill animals.

The dissection of the autoimmune response in TVID did not determine whether the autoimmune responses are an epiphenomenon resulting from tissue damage or whether they actively contribute to the demyelinating process. TMEV may initially infect the CNS bringing about damage to the BBB and other components of the nervous system without eliciting an autoimmune response. As the disease progresses, enough tissue damage and alteration of internal and external self antigens have occurred that the immune system reacts to self. In contrast, autoimmunity in TVID may play a significant role in the initiation, persistence, and

exacerbation of the disease. The virus may target the endothelial cells and smooth muscle cells calling an immune mediated reaction which may produce autoimmunity.

It should be noted that if the whichever theory substantiates the literature suggests that many components, such as the macrophages and T cells, also contribute to the pathogenesis. For example, CD4 T helper cells and CD8 T cytotoxic cells are important initially in viral clearance but later add to the disease ; macrophages participate in bystander demyelination via cytokines from the T cells.

The investigation of the different component of TVID and understanding its pathogenesis may bring about effective methods to treat or prevent multiple sclerosis. Although one can only speculate, there seems to be a menagerie of factors involved in both MS and TVID, such as viral infection, autoimmunity, and humoral and cell-mediated immunity. Effective therapies probably lie in a treating the combination of these areas of the disease.

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