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13. ABSTRACT (Maximum 200 Words)

The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions, host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis we will examine the disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. We will use this system to test the hypothesis that DY TME is not bound by complement resulting in its absence in the spleen. This study will provide details into the host factors(s) involved in transport of prions to cells in the LRS, such as spleen. To date, we have investigated the ability of DY TME to be transported to lymph nodes and if it is present in peritoneal macrophages following intraperitoneal inoculation. We have also are in the process of establishing the ability of cultured macrophages to degrade PrP^{Sc} and the effect of PrP^{Sc} on macrophage survival and phagocytosis. In addition, we have investigated the levels of complement component C1q levels in prion-infected animals.

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Introduction

The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions, host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis two animal models will be used. Genetically engineered mice that lack components of the complement system will be used to test the hypothesis that complement binding to PrP^{Sc} is involved in targeting of prions to cells in the spleen and uptake by macrophages. A second system will examine disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. We will use this system to test the hypothesis that DY TME is not bound by complement resulting in its absence in the spleen. The mouse and hamster systems investigate prion interactions with complement components based on differences of host and strain properties, respectively. This study will provide details into the host factors(s) involved in transport of prions to cells in the LRS, such as spleen.

Body

Tissue distribution of DY TME agent. Animal bioassay was used to investigate if DY PrP^{Sc} is present in spleen, lymph nodes, and peritoneal cells following intraperitoneal inoculation with DY TME as outlined in task 4. Hamsters were intraperitonealy (i.p.) inoculated with DY TME and spleen, mesenteric lymph node, medial iliac lymph node and peritoneal cells (i.e. peritoneal lavage) were collected at from two hamsters at both 60 and 120 days post DY TME i.p. infection. As a negative control, the same tissues were also collected from mock-infected hamsters. The DY TME and mock-infected tissues were homogenized in 100 µl PBS and intracerebrally (i.c) inoculated into four recipient hamsters (25 µl per hamster). As a positive control, hamsters were i.c.

	Incubation Period			
Days post DY TME inoculation	Spleen	Submandibular lymph node	Medial iliac lymph node	Peritoneal cells
60	0/8[a] >400 days	0/8 >400 days	0/8 >400 days	0/8 >400 days
120	0/8 >400 days	0/8 >400 days	0/8 >400 days	0/8 >400 days

[a] Number affected / number inoculated

Table 1. Failure to detect DY TME agent in spleen, lymph nodes and peritoneal cells from DY TME-infected hamsters. Spleen, submandibular lymph node, medial iliac lymph nodes, and peritoneal cells were collected from two hamsters at 60 and 120 days post intraperitoneal DY TME inoculation. The collected tissue was intracerebrally inoculated into 8 recipient hamsters to assay for the presence of DY TME agent.

inoculated with 25 µl of a 10^{-4} dilution of a brain homogenate from a terminally-ill DY TME-infected hamster. The hamsters i.c. inoculated with the DY TME control inoculum had an incubation period of 204±2 days postinfection. The hamsters inoculated with the tissue collected at 60 and 120 days from the DY TME infected hamsters did not result in clinical symptoms of DY TME by 400 days postinfection when the experiment was

terminated (Table 1). Similarly, the tissues collected from the mock-infected hamsters did not cause disease by 400 days postinfection when the experiment was terminated. This data indicates that DY TME is not transported to or replicates in spleen and lymph node. It also indicates that at in peritoneal cells that contain macrophages, the DY TME agent is not detectible suggesting that peritoneal macrophages are not a site of prion competition between HY and DY TME. Earlier time points post DY TME i.p. infection will be investigated to determine if peritoneal macrophages are involved in DY TME transport and/or clearance.

Complement levels in TME-infected brain and spleen. To begin to investigate the interaction of complement with PrP^{Sc} as outlined in task 5, Western blot analysis was used to measure the abundance of C1q in prion-infected brain and spleen and in preparations enriched for HY or DY PrP^{Sc}. Western blot analysis was performed on spleen and brain homogenates from HY TME, DY TME or mock-infected hamsters for the presence of C1q using a polyclonal anti-human C1q antibody (Quidel). Briefly, the tissue homogenates were size fractionated on 12.5% SDS-PAGE and wet transferred to PVDF membrane (Biorad). The membrane was blocked with 5% w/v blotto for 30 minutes at room temperature prior to overnight incubation with the polyclonal anti-human C1q antibody at 4°C (Quidel; 1:1000). Next the membrane was washed and incubated with rabbit anti-goat conjugated to alkaline phosphatase (Biorad; 1:2000) for 1 hour at room temperature. The blot was developed using ECF (Amersham) and imaged using a Molecular Dynamics Storm imaging station and the C1q signal was quantified using ImageQuant software. The banding pattern on the Western blot migrates to the expected molecular weight (Figure 1). The abundance of C1q was similar between HY TME, DY TME and mock-infected brain homogenates (n=3, 1 and 2, respectively) and HY TME and mock-infected spleen homogenates (n= 6 and 4, respectively). To investigate if C1q is directly bound to PrP^{Sc}, PrP^{Sc} enriched preparations from HY and DY TME-infected brains were prepared by detergent extraction and differential centrifugation as previously described (Bartz et al., 2004). The PrP^{Sc} enriched preparations were analyzed for the presence of C1q by Western blot as described previously in this section. C1q was not detected in PrP^{Sc}-enriched preparations from HY or DY TME-infected brain tissue (data not shown). This finding that C1q levels are not increased in brain tissue of TME-infected hamsters is consistent with the observation that i.c. inoculation of complement deficient mice has the same incubation period as wild type mice (Klein et al., 2001; Mabbot et al., 2001). Both pieces of data are consistent with the hypothesis that complement does not contribute to pathogenesis in the central nervous system. The similar abundance of C1q in HY TME-infected spleen compared to mock-infected hamsters suggests that C1q is not involved in HY TME replication in the spleen and suggests that the increase in incubation period in complement deficient mice inoculated i.p. is due to prion opsonization and transport to the spleen.

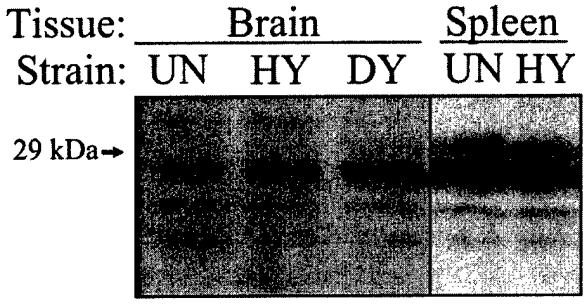

Figure 1. Western blot analysis of C1q abundance in uninfected (UN), hyper TME infected (HY) and drowsy TME infected (DY) hamster brain and spleen. The migration of the 29 kDa molecular weight marker is indicated on the left of the panel.

Serum amyloid protein (i.e. Female protein) levels in prion-infected hamsters. It is possible that the observed increase in incubation period in complement deficient animals is not

due to a direct interaction of complement components with PrP^{Sc} but via an intermediate molecule. A possible candidate molecule is serum amyloid protein (SAP) that has been shown to bind to amyloid and can also directly bind to C1q (Coe and Ross, 1990; Nauta et. al., 2003). To begin to investigate this possibility, we have obtained a polyclonal serum to female protein (FP), the hamster homolog of SAP, from Dr. John Coe (NIH, Rocky Mountain Laboratories). We have initiated experiments using this serum to determine the levels of FP in prion-infected hamster brain and spleen. While there are no differences in migration or abundance of FP in uninfected and HY TME-infected spleen (Figure 2), a difference in the banding pattern of FP was observed in HY TME-infected brain compared to an uninfected control (Figure 2). We are currently examining additional uninfected, HY TME and DY TME-infected brain homogenates to further investigate this observation. Subsequent studies using this antibody will investigate the ability of purified HY and DY PrP^{Sc} to bind FP using the same methodology used to investigate C1q binding to PrP^{Sc} as outlined in task 5.

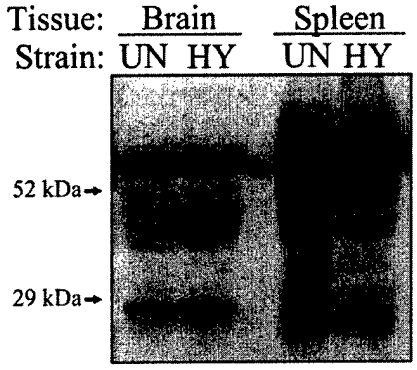

Figure 2. Western blot analysis of female protein abundance in uninfected (UN) and hyper TME infected (HY) hamster brain and spleen. The migration of the 52 and 29 kDa molecular weight markers are indicated on the left of the panel.

Antibody response to DY PrP^{Sc}. Recent reports have suggested that antibodies directed against PrP can increase the incubation period of prion disease (Schwarz et al., 2003; Sigurdsson et al., 2003; Beringue et al., 2004). This suggested to us that a possible mechanism for the ability of DY TME to extend the incubation period of superinfected HY TME following i.p. inoculation was due to generation of antibodies against DY PrP^{Sc} that would cross react with HY PrP^{Sc}. To investigate if antibodies are generated against DY PrP^{Sc}, we developed a 96 well immunoassay to detect PrP specific antibodies.

Briefly, an Acrowell 96 Filter plate with an attached polyvinylidene difluoride (PVDF) membrane (Pall Life Sciences, Ann Arbor, MI) was activated with methanol (Fisher Scientific, Pittsburg, PA). The plate was then washed and centrifuged for 1 minute at 1500 x g with TTBS (10 mM Tris-HCl (pH 7.4) 150 mM NaCl, 0.5% Tween-20). The samples (250ug equivalents of brain homogenate from uninfected, HY TME or DY TME-infected hamsters) were then dispensed into the plate. The plate was centrifuged for 1 minute at 1500 x g. The plate was washed and centrifuged for 1 minute at 1500 x g. Endogenous perioxidases were blocked with a 20 minute incubation in 0.3% hydrogen peroxide (Sigma, St. Louis, MO) in methanol. The plate was again washed and centrifuged for 1 minute at 1500 x g. The samples were either denatured by treatment with 3M guanadinium thiocyanate (Fluka Biochemika/Sigma, St. Louis, MO) for 10 minutes for detection of linear epitopes or treated with TTBS for detection of epitopes that may be present on non-denatured PrP. The plate was washed and centrifuged again prior to a 30 minute incubation at 37°C in 5% nonfat dry milk blocking solution (Bio-Rad, Hercules, CA). The plate was incubated undiluted serum from uninfected or DY TME infected hamsters for 1 hour at 37°C. The plate was washed and centrifuged for 1 minute at 1500 x g prior to incubation with a peroxidase conjugated rabbit-anti hamster immunoglobulin G (Jackson laboratories). The plate was then washed and centrifuged for 1 minute at 1500 x g prior to development with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and visualized with a Kodak Image Station 2000R in luminescence mode.

Using this method we have plated brain tissue homogenized in the presence of protease inhibitors or proteinase K digested from at least 3 individual uninfected, DY TME and HY TME-infected hamsters. Using these tissues as the "antigen" we have investigated for the presence of antibodies in serum from 6 uninfected animals as a negative control and at least three individual hamsters infected with DY TME by either the intracerebral, intraperitoneal or intralymph node routes of infection. We also investigated for the presence of antibodies in serum from three hamsters intracerebrally infected with HY TME. Using these combinations of brain tissue and serum we were unable to identify an antibody response in HY TME or DY TME-infected hamsters. This is a significant finding and excludes the possibility that the extension of superinfected HY TME is due to an antibody response to PrP or that antibody opsonization of PrP^{Sc} facilitates prion transport.

Macrophage degradation of TME PrP^{Sc}. The interactions of HY and DY TME PrP^{Sc} and macrophages are being investigated as outlined in task 6. Significant progress has been made on co-culture parameters such as cell viability and PrP^{Sc} analysis from cell culture. In addition, a recent report has demonstrated that a synthetic peptide to PrP and PrP^{Sc} can inhibit phagocytosis in microglia (Cieseilski-Treska et al., 2004). Therefore, we have also included a phagocytosis assay to determine if exposure to PrP^{Sc} inhibits the ability of macrophages to phagocytose. It is possible that strain-specific inhibition of macrophage phagocytosis could explain the lack of detection of DY PrP^{Sc} in peritoneal cells and secondary lymphoreticular system tissues. In the original proposal, enrichment of PrP^{Sc} by detergent extraction and ultracentrifugation followed by Western blot analysis was to be used to quantify PrP^{Sc} degradation following co-culture with macrophages. However, due the large number of samples involved we investigated alternative methods to decrease the amount of time required to assay for PrP^{Sc} abundance. We have developed an immunoassay that is based on a 96 well filter plate that is nearly as sensitive as Western blot analysis and can be completed in 4-6 hours. We are continuing to develop this system with the goal of increasing sensitivity to reduce the numbers of animals that we will use for animal bioassay of prions. The methodology for these studies is outlined below. We are currently using these methods to determine the kinetics of PrP^{Sc} degradation by macrophages and the effect of PrP^{Sc} on macrophage viability and phagocytosis capacity.

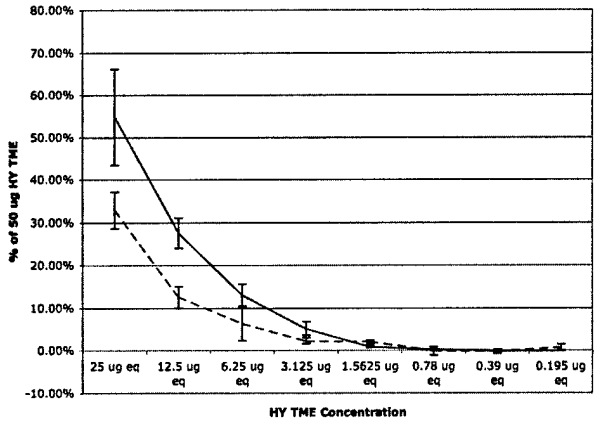

Figure 3. Western blot (solid line) and 96 well plate assay (dashed line) of HY PrP^{Sc}. Two fold serial dilutions of proteinase K digested HY TME brain homogenates were analyzed by Western blot and 96 well assay in triplicate as described in the text. The signal strength of PrP^{Sc} is represented as a percentage of 50ug of HY TME brain equivalents. The minimum detectable signal is calculated to be 3.125ug equivalents and 1.5625ug equivalents for the Western blot and 96 well assay respectively.

PrP^{sc} Analysis: PrP detection was done by 96-well immunoassay. An Acrowell 96 Filter plate with an attached polyvinylidene difluoride (PVDF) membrane (Pall Life Sciences, Ann Arbor, MI) was activated with methanol (Fisher Scientific, Pittsburg, PA). The plate was then washed and centrifuged for 1 minute at 1500 x g with TTBS (10 mM Tris-HCl (pH 7.4) 150 mM NaCl, 0.5% Tween-20). The samples were then dispensed into the plate. The plate was then centrifuged for 1 minute at 1500 x g. The plate was then washed and

centrifuged for 1 minute at 1500 x g. Endogenous perioxidases were saturated with a 20 minute incubation in 0.3% hydrogen peroxide (Sigma, St. Louis, MO) in methanol. The plate was again washed and centrifuged at 1500 x g for 1 minute. The samples were then denatured by treatment with 3M guanadinium thiocyanate (Fluka Biochemika/Sigma, St. Louis, MO) for 10 minutes at room temperature. The plate was washed and centrifuges at 1500 x g for 1 minute prior to 30 minute incubation at 37°C in 5% nonfat dry milk blocking solution (Bio-Rad, Hercules, CA). The plate was incubated with the monoclonal 3F4 ascites fluid (1:10,000; a gift from Richard Kascsak New York State Institute of Mental Health, Staten Island) for 1 hour at 37°C. The plate was washed and centrifuged at 1500 x g for 1 minute prior to incubation with a peroxidase conjugated goat-anti mouse immunoglobulin G (1:2000; Pierce, Rockford, IL). The plate was then washed and centrifuged for 1 minute at 1500 x g prior to development with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and visualization on a Kodak Image Station 2000R in luminescence mode.

Cell viability: Cells were assessed for viability by incubating the cells in either RPMI media without phenol red (Biosource, Rockville, MD) or DMEM without phenol red (Biosource, Rockville, MD) with 2 ug/ml Fluorescein Diacetate (Molecular Probes, Eugene, OR) and 50 ug/ml of Propidium Iodide (Molecular Probes, Eugene, OR) for 10 minutes. The cells were then view at 4x on a Nikon fluorescent microscope with a dual FITC/TRITC filter.

Macrophage phagocytosis: Normal macrophage function was assessed by measuring the ability of the cells to phagocytose fluorescent bioparticles. This assessment was done with the Vybrant Phagocytosis Assay Kit (Molecular Probes, Eugene, OR) per the manufacturer's instructions.

Key Research Accomplishments

1. Bioassay of spleen, mesenteric lymph node, medial iliac lymph node, and peritoneal cells from hamsters i.p. inoculated with DY TME at 60 or 120 days post infection have been unable to detect DY TME infectivity.
2. Western blot analysis of C1q abundance in spleen and brain from HY and DY TME-infected hamsters is not altered compared to mock-infected hamsters.
3. Western blot analysis of female protein in brain demonstrates differences in banding pattern in HY TME-infected hamsters compared to uninfected control animals.
4. Conditions for quantifying PrP^{Sc} abundance, macrophage cell viability and macrophage phagocytosis capability have been established in macrophage cells incubated with HY and DY TME PrP^{Sc}.

Reportable Outcomes

None

Conclusions

At 60 and 120 days post intraperitoneal infection with DY TME, the DY TME agent is not present in the spleen, mesenteric lymph node, medial iliac lymph node, and peritoneal cells. This data is consistent with the hypothesis that DY TME is a lymphoreticular system replication deficient prion strain. The levels of C1q are unchanged in brain and spleen of hamsters infected with HY and DY TME compared to mock infected controls. This data indicates that elevated levels of C1q is not involved in

prion replication and is consistent with previous studies that demonstrate in C1q deficient animals intracerbral infection has an incubation period similar to wildtype controls (Klein et. al., 2001; Mabbot et. al., 2001). This data does not exclude the possibility that C1q is involved in prion transport to sites of replication. As an alternative mechanism, the observed increase in incubation period in complement deficient animals may not due to a direct interaction between complement and PrP^{Sc} but could be mediated by an intermediate molecule. To investigate this possibility, we have initiated experiments examining the abundance of female protein in hamsters, the hamster homolog of serum amyloid protein, and have evidence that in HY TME-infected animals FP expression is altered. In addition, we have modified and optimized the culture conditions for the analysis of PrP^{Sc} degradation by macrophages.

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Appendices

None