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Award Number: DAMD17-03-1-0319

TITLE: Prion Transport to Secondary Lymphoreticular System Tissues

PRINCIPAL INVESTIGATOR: Jason C. Bartz, Ph.D.

CONTRACTING ORGANIZATION: Creighton University
Omaha, NE 68178-0001

REPORT DATE: June 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-06-05	Annual	06/01/04-05/31/05

4. TITLE AND SUBTITLE
Prion Transport to Secondary Lymphoreticular System Tissues

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0319

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jason C. Bartz, Ph.D.

Email-jbartz@creighton.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Creighton University
Omaha, NE 68178-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

Abstract can be found on next page.

15. SUBJECT TERMS
prion diseases, macrophage, complement

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)* 301-619-7325

ABSTRACT

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. We have shown differences in the migration of female protein, the hamster homologue of serum amyloid protein, in TME-infected hamsters. We can selectively immunoprecipitate PrP^{Sc} for experiments to determine if PrP^{Sc} is opsonized. We also have preliminary data suggesting differences in the susceptibility of HY and DY TME to phagocytosis and degradation by macrophages.

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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 HY & DY TME at early time points post-infection. To investigate the disribution of infectivity and PrP^{Sc} in hamsters infected with HY and DY TME at early time points post-infection as outlined in task 4, hamsters were intraperitoneally inoculated with uninfected homogenate or $10^{4.5}$ LD_{50} of DY TME or $10^{7.5}$ LD_{50} of HY TME. At 7.5 hours, 2.5, 5, 10, 20 and 40 days post-infection, three animals for each inoculation group were sacrificed and peritoneal cells, spleen, mesenteric lymph node, medial iliac lymph node and submandibular lymph node were collected. We are currently using detergent extraction and ultracentrifugation to enrich for PrP^{Sc} from one-half of each sample prior to Western blot analysis as previously described (Bartz et al., 2004). Samples that are negative for PrP^{Sc} by Western blot will be intracerebrally inoculated into hamsters to determine the samples contain the HY or DY TME agent.

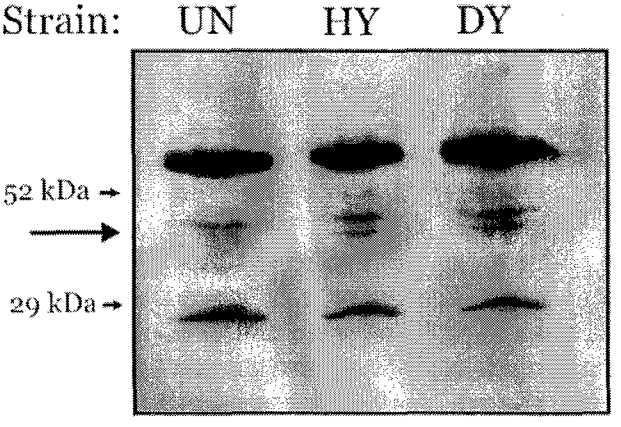

Figure 1. Differences in female protein migration patters from uninfected (UN), HY TME-infected (HY) and DY TME-infected (DY) brain homogenate. Migration of the 52 and 29 kDa molecular weight markers are indicated on the left of the blot. The arrow indicated the migration of the additional band that is present in the HY and DY TME-infected brain homogenates.

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). For these experiments, 1000 ug equivalents of uninfected, HY or DY TME-infected 10% w/v brain homogenates from 6 individuals from each group containing protease inhibitors (Roche Diagnostics, Mannhiem, Germany) were size fractioned on 12.5% SDS-PAGE and transferred to Immobilon P as previously described (Bartz et al., 2004). The membrane was incubated with the rabbit anti-female protein in TTBS containing 5% Blotto (Bio-Rad Laboratories, Hercules, CA) overnight at 4°C. The membrane was washed with TTBS and incubated with a 1:100 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (Pierce, Rockford, IL). The Western blot was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) according to manufactures instructions. The chemiluminescence signal was detected using a Kodak 2000R imaging station (Kodak, Rochester, NY).

This experiment was repeated with six different uninfected, HY and DY-infected brain homogenates (Figure 1). An addition band was present in all of the HY and DY-infected hamsters and the abundance of the addition band was similar between hamsters infected with HY or DY TME (Figure 1). We are currently determining female protein abundance and banding patterns in spleen and lymph nodes from uninfected, HY and DY TME-infected hamsters.

Response of female hamsters to DY TME-infection. In hamsters, female protein is regulated by estrogen resulting in approximately 100-fold higher serum level of female protein compared to male hamsters (Coe & Ross, 1990). To investigate if this higher level of serum protein had an effect on DY TME incubation period and DY PrP^{Sc} properties, male and female hamsters were intracerebrally inoculated with $10^{3.8}$ LD_{50} of DY TME. The hamsters were assessed daily for the onset of clinical symptoms of DY TME which is characterized by a progressive lethargy. Male hamsters had an incubation period of 169±3 days (±SEM) while the female hamsters had an incubation period of 174±4 days. The incubation period of DY TME in male and female hamsters was not statistically significant ($p>0.05$ Student's T-test). This differs from previous studies that indicated in an eight percent reduction in the incubation period of hamsters i.c. inoculated with the 263K strain of hamster-adapted scrapie (Kimberlin & Walker, 1977) but is consistent with studies in mice where the incubation period in females is not uniformly shorter than in males (Outram, 1976).

DY PrP^{Sc} from the female hamsters will be used in task 5 and 6 to determine if the increased levels of female protein affect the ability of PrP^{Sc} to bind female protein and if it effects the ability of PrP^{Sc} to be phagocytosed by macrophages.

Immunoprecipitation of HY and DY PrP^{Sc} and quantification of remaining PrP^{C}. To identify factors that may bind to and opsonize PrP^{Sc}, we are immmunoprecipitating PrP^{Sc} followed by Western blot analysis for potential candidates that may bind PrP^{Sc} (e.g. female protein, C1q) as outlined in task 5.

For immunoprecipitation of PrP^{Sc}, M-450 rat anti-mouse IgM dynabeads (Dynal biotech, Oslo Norway) are prepared for immunoprecipitation according to manufactures instructions and are incubated with the 15B3 antibody (a generous gift from Alex Rabier and Bruno Oesch, Prionics AG, Switzerland; Nazor et al., 2005) for 2 hours at room temperature with gentle shaking. After incubation, the beads are placed on a magnetic particle concentrator (MPC-S; Dynal Biotech, Oslo, Norway) and the supernatant containing unbound 15B3 is removed and discarded. The beads are washed by the addition of five volumes of coating buffer [0.1% v/w BSA (Sigma-Aldrich, St. Louis, MO) in DPBS (Mediatech, Inc., Herdon, VA)] followed by vortexing. The beads are concentrated using the MPC-S and the supernatant is removed. After three washes, the beads are resuspended in one volume of coating buffer and stored at 4°C. To 30 µl of the prepared beads, 250 µg brain equivalents of brain homogenate containing protease inhibitors (complete protease inhibitors – Roche Diagnostics GmbH, Manheim, Germany) and 445 µl of immunoprecipitation buffer (0.3% sarkosyl in TBS) are added and incubated at room temperature with gentle shaking for two hours. The 15B3 coated magnetic beads that has bound PrP^{Sc} are collected with the MPC-S magnet and the supernatant containing PrP^{C} is collected. The supernatant is centrifuged at 45,000 RPM in a TLA-45 rotor (Beckman) for 1 hour at 10°C. After centrifugation, the supernatant is discarded and the pellet is resuspended in 10 µl of DPBS (Mediatech, Inc., Herdon, VA). Half of the PrP^{C} containing samples is digested with 4 units/ml of PK (Roche Diagnostics) at 37°C for one hour while the remaining half is left undigested. To the beads containing PrP^{Sc}, 1 ml of wash buffer (0.25% sarkosyl in TBS) is added. The magnetic beads are then vortexed and collected using the MPC-S magnet. The supernatant is removed and discarded. The magnetic beads containing PrP^{Sc} are resuspended in 50 µl DPBS and are digested with 2 U/ml Proteinase K (Roche Diagnostics) at 37°C for one hour. The PK digestion is stopped by the addition of pefabloc (Roche Diagnostics) to a final concentration of 2 mM. After addition of 1ml wash buffer, the PK digested PrP^{Sc} bound to the magnetic beads via 15B3 is collected using the MPC-S magnet. The supernatant is removed and discarded. The PrP^{Sc} is separated from the beads by boiling in 25 µl of SDS-PAGE loading buffer for five minutes. The beads are collected with the MPC-S magnet and the supernatant containing PrP^{Sc} is removed and analyzed by Western blot using the anti-PrP monoclonal antibody, 3F4 as previously described (Bartz et al., 2004).

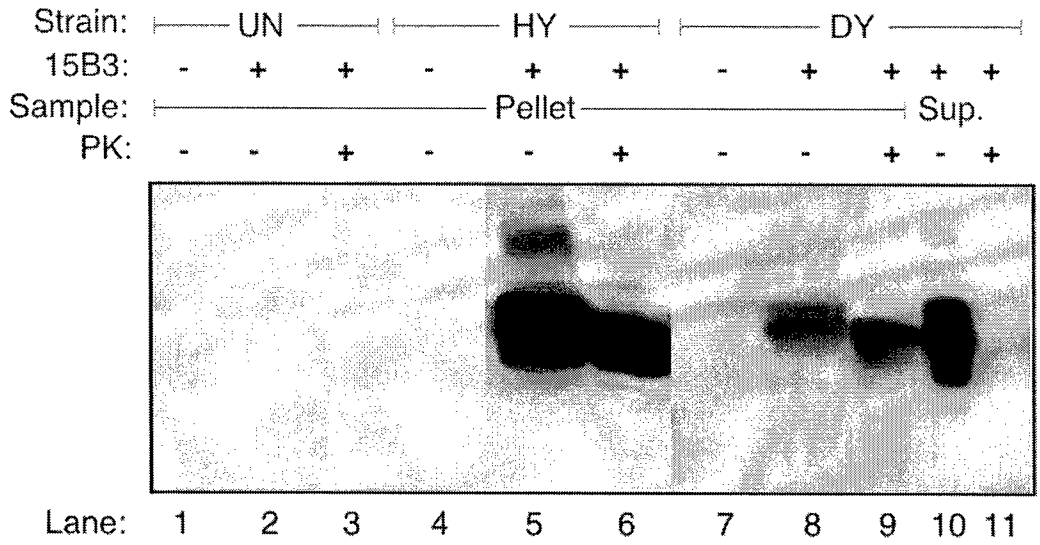

Figure 2. Immunoprecipitation of HY and DY PrP^{Sc} from brain tissue. The anti-PrP^{Sc} antibody 15B3 does not immunoprecipitate PrP^{C} (lanes 1-4; 7) but proteinase K (PK) resistant PrP is immunoprecipitated from HY TME (lanes 5 &6) and DY TME-infected brain material (lanes 8 & 9). Following the immonoprecipitation of PrP^{Sc} from DY TME-infected brain material, the supernatant was collected and shown to contain PK sensitive PrP (lanes 10 &11).

Using this system, we can immunoprecipitate PK resistant PrP^{Sc} from HY and DY TME-infected brain material (Figure 2, lanes 5 & 8) but PrP^{C} is not immunoprecipitated from uninfected hamster brain (Figure 2, lane 2) indicating that 15B3 is specific for PrP^{Sc} isoform of the prion protein. The PrP^{Sc} that is immunoprecipitated from HY and DY TME-infected brain is resistant to PK digestion (Figure 2, lanes 6 & 9). An additional control to indicate the specificity of the immunoprecipitation for PrP^{Sc} is the omission of the 15B3 antibody, which results in a failure to immunoprecipitate PrP^{Sc} (Figure 2, lanes 4 & 7). In DY TME infected hamster brain, when PrP^{Sc} is immonoprecipitated with 15B3 and the supernatant is collected and analyzed by Western blot, it is shown that the supernatant contains PrP (Figure 2, lane 10) that is sensitive to PK digestion (Figure 2, lane 11).

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 co-culturing HY and DY TME with the murine macrophage cell line RAW 264.7. In these experiments, RAW 264.7 cells are grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM, ATCC, Manassas, VA) containing 5% v/v fetal bovine serum (ATCC, Manassas, VA) 1mM L-glutamine (Gibco) and 100 U/ml of penicillin and 100 µg/ml streptomycin (Sigma). The cells were removed from the flask by scraping, and the number of RAW 264.7 cells was estimated using a hemocytometer (Hausser Scientific, Horsham, PA) and the cell concentration was adjusted to 10^6 cells per ml using pre-warmed (37°C) DMEM.

To assess if RAW 264.7 cells could degrade HY or DY PrP^{Sc}, PrP^{Sc} was incubated with either 10^5 RAW 264.7 cells or an equal volume of DMEM without cells as a control for non-RAW 264.7 mediated PrP^{Sc} degradation. Following addition of the RAW 264.7 cells, the plate was incubated for 1 hour at 37°C to allow for cell adhesion. The media was removed and replaced with DMEM that contained PrP^{Sc}. The PrP^{Sc} that was tested was from either a DY TME brain homogenate, a HY TME brain homogenate, a DY PrP^{Sc} enriched preparation or a HY PrP^{Sc} enriched preparation. The brain homogenates were prepared in PBS and were not digested with proteinase K, and PrP^{Sc} was enriched from HY or DY-infected brain using detergent extraction and ultracentrifugation as previously described (Bartz et al., 2004). Following the 1 hour incubation, the media was removed and to each well containing either 10^5 RAW 264.7 cells or DMEM, 100 µg equivalents of TME-infected brain homogenate or a volume of PrP^{Sc}-enriched preparation that contained an equal amount of PrP^{Sc} as the brain homogenate (as determined by Western blot) was added. Samples in triplicate were collected at 0, 24, 48 and 72 hours after TME-infection. At each collection point the media was removed and saved. An equal volume of DMEM was added to the well to collect the remaining free PrP^{Sc} and was added to the previously collected media. The PrP^{Sc} in the media and wash is referred to as the media associated PrP. To the cells, 100µl of 0.1% w/v NLS was added to the well to dislodge the cells from the well and collected. The wells were then washed with 100 µl of

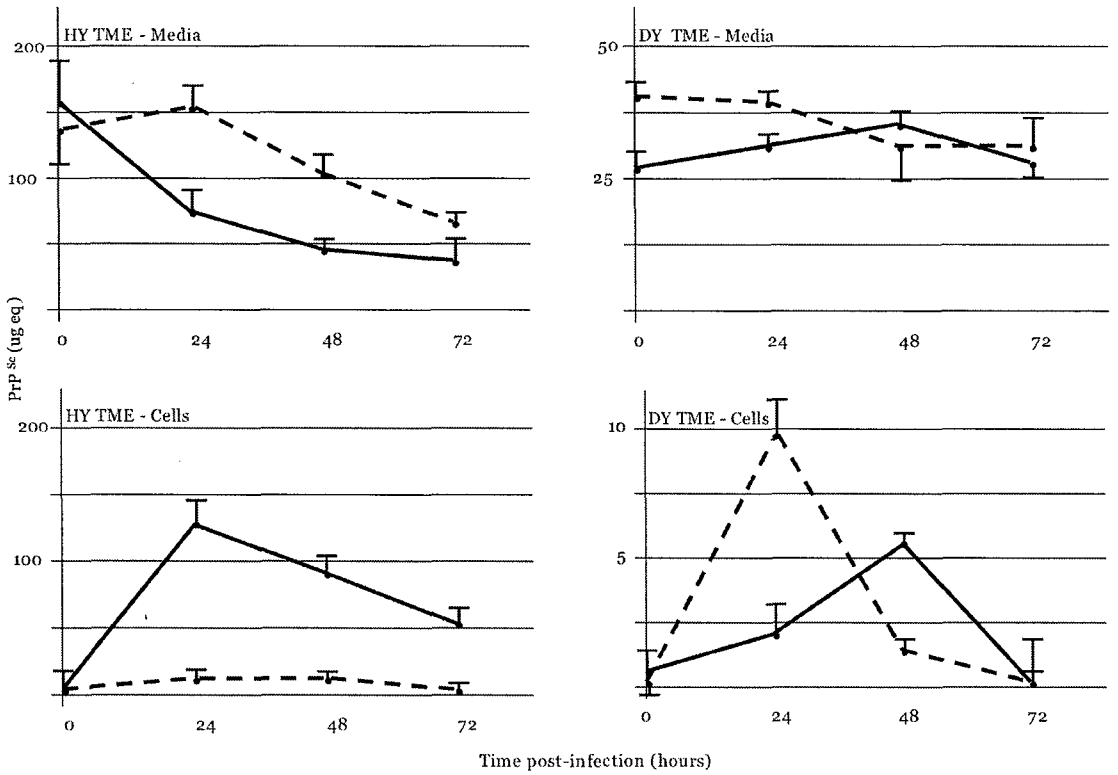

Figure 3. Abundance of HY or DY PrP^{Sc} (ug/ml) in the media or associated with macrophages (cells) at selected time points (0, 24, 48 & 72 hours) post infection.

0.1% w/v NLS that was added to the first cell collection. The PrP^{Sc} collected in 0.1% NLS is referred to as cell-associated PrP. The levels of PrP in the media and associated with the cells were quantified using a 96 well immunoassay that detects PrP using the anti-PrP monoclonal antibody, 3F4.

Using this system, we have identified strain-specific differences in the ability of macrophages to degrade PrP^{Sc}. In HY TME-infected macrophages with brain homogenate or PrP^{Sc}-enriched preparation there was a reduction in PrP^{Sc} levels in the media at 48 and 72 hours post-infection although there was a greater reduction in PrP^{Sc} from the enriched preparation (Figure 3). In macrophages infected with HY TME brain homogenate, the cell associated PrP^{Sc} level was undetectable at T=0, increased at 24 and 48 hours post-infection and at 72 hours post-infection PrP^{Sc} levels were again undetectable. This was in contrast to HY PrP^{Sc}-enriched preparation infected cells where at 24 hours post-infection there was a large amount of PrP^{Sc} associated with the cells that was reduced in abundance but not totally cleared from the cells at 72 hours post-infection (Figure 3). In DY infected macrophages there no reduction in the levels of PrP^{Sc} in the media from macrophages infected with DY brain homogenate or DY PrP^{Sc}-enriched preparation (Figure 3). In macrophages infected with DY TME-infected homogenate, cell-associated PrP^{Sc} was first detected at 24 hours post-infection and at later time points PrP^{Sc} levels were reduced and were undetectable at 72 hours post-infection. In macrophages infected with DY PrP^{Sc} from an enriched preparation, PrP^{Sc} was detected at 24 hours post-infection, peaked at 48 hours post-infection and were undetectable at 72 hours post-infection (Figure 3).

To summarize the differences between HY and DY TME-infected macrophages, HY PrP^{Sc} from enriched preparations associates with the macrophages at a higher level than DY TME, and the levels of HY PrP^{Sc} from enriched preparations is detectable at time points where DY PrP^{Sc} from enriched preparations has be degraded. This suggests that HY TME may be more resistant to degradation, is would not be cleared from the host unlike DY TME. In addition the lower levels of HY PrP^{Sc} in the media compared to DY TME suggests that HY TME is more efficiently phagocytosed than DY TME. Combined with the seeming reduction in clearance of HY TME by macrophages suggests that macrophages may be a cell type that can carry PrP^{Sc} to spleen and lymph nodes.

In both TME strains, the degradation of PrP^{Sc} from enriched preparations and brain homogenates differed (Figure 3). This suggests that either the physical properties of PrP^{Sc} are changed in the enriched preparation or that cytokines and/or chemokines that are present in the brain homogenates are influencing the properties of the cells.

Experiments are underway, to determine the kinetics of media and cell-associated PrP^{Sc} at time points prior to 24 hours and to determine in macrophages infected with HY PrP^{Sc} from enriched preparations when PrP^{Sc} is cleared from the cells.

Key Research Accomplishments

1. Peritoneal cells and lymphoreticular system tissues from uninfected, HY TME and DY TME-infected hamsters have been collected and we are in the process of determining the spatial and temporal spread of PrP^{Sc} in these tissues.
2. The presence of differential banding pattern of female protein in both HY and DY TME-infected brain compared to uninfected control brain material has been verified.
3. Determined that female hamsters are susceptible to intracerebral DY TME infection with the same incubation period as male hamsters.
4. Demonstrated selective immunoprecipitation of HY and DY PrP^{Sc} from brain homogenates to be used in pull down experiments to investigate if PrP^{Sc} binds opsonins (e.g. C1q)
5. Co-culture experiments of macrophages and PrP^{Sc} from HY and DY TME-infected hamsters demonstrated a strain specific response of PrP^{Sc} degradation.

Reportable Outcomes

None

Conclusions

We have initiated studies on the tissue distribution of HY and DY TME at early time points post intraperitoneal infection. We have collected peritoneal cells and lymphoreticular system tissues and are in the process of analyzing these tissues for the presence of PrP^{Sc}. We have demonstrated that female hamsters, that express higher levels of female protein than male hamsters, are susceptible to intracerebral inoculation with DY TME and the incubation period is not different that male hamsters intracerebrally inoculated with the same dose of DY TME. We are planning on using the PrP^{Sc} from these animals for macrophage phagocytosis assays and to determine if female protein is associeated with PrP^{Sc}. We have shown that HY and DY PrP^{Sc} can selectively be immunoprecipitated from brain tissue. We are currently using this technique to immunoprecipitate PrP^{Sc} and perform Western blot analysis for molecules that we hypothesize bind to PrP^{Sc} (e.g. C1q). Finally, we have evidence of strain-specific phagocytosis and degradation of PrP^{Sc} by macrophages.

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Appendices

None