Determining a role for microglia in PrP^{SC}-mediated pathogenesis *in vitro* and *in vivo*

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Specific Aims

To understand the role of microglia in prion disease pathogenesis *in vitro* and *in vivo*, we propose the following aims:

Aim 1. Determine the cell types sufficient to mediate PrP^{SC}–induced neuronal toxicity via release of diffusible factors *in vitro*.

Aim 2. (a) Generate transgenic mouse with inducible ablation of microglia, monocytes, and macrophages and characterize mouse phenotype after loss of these cell types.

(b) Determine whether microglia are necessary for prion disease pathogenesis *in vivo*.

Background and Significance

The transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a family of rare, fatal neurodegenerative diseases affecting humans and several other species of mammals. These diseases are unique in that they can be both inheritable and infectious. TSEs include scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle as well as iatrogenic, sporadic, and familial forms in human (e.g. Creutzfeldt-Jakob (CJD, vCJD), Kuru, Gerstmann-Sträussler-Scheinker syndrome, Fatal Familial Insomnia). Despite a wide variety of symptoms ranging from dementia to ataxia to insomnia, the pathology of all of the TSEs is characterized by neuronal loss, gliosis, and vacuolation of neurons and neuropil called spongiform change (Prusiner, 1996). Amyloid plaque formation may also occur.

Although the prion diseases are very rare, there is great interest in their neuropathology due to both the unique biological attributes of the infectious particle and the public health issues of BSE and CJD transmission in humans. After much controversy, the infectious agent in these disorders is widely believed to be a protein, now termed a prion (from *proteinaceous infection only*) (Glatzel & Aguzzi, 2001). Unlike other infectious agents, the only particle required for transmission and progression of infection in TSEs is the protein. Standard sterilization procedures have little or no effect on prions, increasing the risk of iatrogenic infection (Diringer, 1999).

The normal cellular prion protein, PrP^{C}, is a glycosyl-phosphatidylinositol-anchored protein that is expressed in neurons, glia, immune cells, and muscle cells (Brown *et al.*, 1990; Bendheim *et al.*, 1992). PrP^{C} has been implicated in an anti-apoptotic pathway, but the normal protein's exact function remains uncertain (Rezaie & Lantos, 2001). When PrP^{C} comes into contact with the infectious form, PrP^{SC}, a conformational change occurs turning the endogenous PrP^{C} into PrP^{SC} (Jackson, 2001). The PrP^{SC} protein has much more beta-sheet structure, making it insoluble and much less resistant to protease digestion (Prusiner, 1998).

The causes of the neurodegenerative changes that follow prion infection are unclear. Loss of PrP^{C} through conversion to PrP^{SC} is not neurotoxic in itself, as knockout $PrP^{C-/-}$ mice develop normally (Brander *et al.*, 1996). In addition, PrP^{SC} is not directly toxic to the neurons, as PrP^{SC} neurotoxicity is dependent upon the presence of PrP^{C}; the knockout mice lacking PrP^{C} are resistant to prion propagation and disease development even when administered continuous, high doses of PrP^{SC} (Büeler *et al.*, 1993, Brander *et al.*, 1996). Neurotoxicity does occur, both *in vitro* and *in vivo,* following PrP^{SC} infection of $PrP^{C-/-}$ neurons interacting with wild type, PrP^{C} -expressing astrocytes (Raeber *et al.*, 1997; Brown, 1999). In these cases, only neurons lacking PrP^{C} die, possibly due to an increased susceptibility of the neurons to toxic stresses and a loss of the normal protective effects of astrocytes for glutamate toxicity. Consequently, neuronal interactions with surrounding glia are now thought to play a key role in PrP^{SC} pathogenesis.

Recently, attention has focused on microglia in prion neuropathogenesis. It was found that not only do microglia kill prion-infected neurons *in vitro*, but even just media from cultures of microglia activated by PrP^{SC} can induce neurotoxicity in wild type cultures (Bate *et al.*, 2001; Combs *et al.*, 1999). Microglia are phagocytic

cells that develop from the monocyte cell line and serve as an extension of the immune system in the brain. In the healthy adult brain, microglia are in a quiescent state. They may continue to release neurotrophic factors in the healthy brain, but are primarily active during development and disease. When an infectious particle is detected, microglia become activated, releasing matrix-degrading enzymes, reactive oxygen species and cytokines such as Il-1, Il-6, and TNF-β, which produce a localized inflammatory response and recruit macrophages from the blood stream (Barron, 1995). In prion encephalopathies, these macrophages may actually be the route by which PrP^{SC} invades the brain (Mabbott & Bruce, 2001). While the prion protein does not induce a systemic inflammatory reaction, accumulating evidence suggests that such localized inflammatory processes mediated by microglia are also important in the neuropathogenesis of HIV and Alzheimer's disease (McGeer & McGeer, 1998; Adle-Biassette, *et al*., 1999).

Thus neurodegeneration may occur because of two concurrent factors. First, neurons may become more susceptible to toxic insults following infection with PrP^{SC}. Second, an increase in toxic substances may hasten neuronal death. Microglia appear to be in a key position to mediate this neuronal toxicity. Microglia activation parallels PrP^{SC} deposition spatially and temporal and precedes neurodegeneration *in vivo* (Geise *et al.*, 1998; Williams *et al.*, 1997). Furthermore, cytokine release by microglia is necessary for the proliferation of astrocytes *in vitro* (Hafiz, 2000). Thus microglia may not only produce toxic compounds that precipitate neuronal death and spongiform change, but may also induce the gliosis seen in these diseases.

While microglia are clearly involved in the pathogenesis of prion diseases, we do not yet know exactly what cells they interact with nor whether microglia play a primary or secondary role, as the function of microglia during PrP^{SC} infection has not yet been studied *in vivo*. We propose to first investigate what cell types are sufficient to induce neurotoxicity *in vitro* following treatment with PrP^{SC}. Next, we plan to examine the role of microglia in prion pathogenesis by knocking out microglia, macrophages, and monocytes in mice at various time points during PrP^{SC} infection. If activated microglia are crucial for the *in vivo* PrP^{SC}-mediated pathogenesis, suppression of the activation of microglia could be used in the treatment of prion diseases.

Research Design and Methods

Aim 1. *Determine the cell types sufficient to mediate PrP^{SC}–induced neuronal toxicity via release of diffusible factors in vitro.*

Experimental Design. Previous experiments have shown that various cell types are involved during prion pathogenesis (Brown *et al.*, 1996; Race *et al.*, 1995; Raeber *et al.*, 1997; Rezaie and Lantos, 2001). We also know that cells communicate to one another via a variety of diffusible factors (such as cytokines, chemokines, and growth factors), many of which are upregulated during prion pathogenesis (Rezaie and Lantos, 2001). Additionally, conditioned medium from prion-stimulated microglia has been shown to be neurotoxic to neuronal cultures (Combs *et al.*, 1999). Therefore, we propose a series of *in vitro* culture experiments to identify the major cell types in the mammalian brain that are sufficient to mediate PrP^{SC}-induced toxicity via release of diffusible factors.

In the first set of experiments, cultures of specific WT (PrP^{C} -expressing) mouse cell types will be prepared: 1) cortical neurons from E18 mice, 2) astrocytes from P0 mice, and 3) microglia from P0 mice. Cells will be isolated and cultured following protocols modified from Goslin *et al.* (1998) and Brown *et al.* (1996). Additionally, conditioned media will be used to substitute for cell types absent from a specific culture condition: most importantly, neurons will be grown in astrocyte- and microglia-conditioned media to provide trophic support (Ullian *et al.*, 2001). After 7 days in vitro (DIV), cultures will be treated with PrP^{SC} or BSA (as a control) according to established protocols (Mueller *et al.*, 1993; Giese *et al.*, 1995; Ushijima *et al.*, 1999). After an additional 12 to 24 hours, the response of each cell type will be characterized by assaying for general changes in morphology, and, as appropriate, neuronal death using a cell death assay (as described in Brown *et al.*, 1996 and Deli *et al.*, 2000), microglia activation using antibody staining for markers of activation (described in Rezaie and Lantos, 2001), or glial proliferation using traditional cell counting techniques or by counting the number of BrdU-labeled nuclei (as described in Fischer and Reh, 2001).

In the second set of experiments, the conditioned media from the PrP^{SC}- or BSA-treated cell-type specific cultures will be purified on an anti-PrP^{SC} column to remove PrP^{SC}. Full removal of PrP^{SC} will be verified by Western blot. The three cell-type specific conditioned media will then be applied to separate cultures (after 7

DIV) containing 1) cortical neurons, 2) cortical neurons with astrocytes, 3) cortical neurons with microglia, and 4) cortical neurons with astrocytes and microglia (for a total of 12 experimental and 12 control culture conditions). Again, conditioned media will be used to substitute for cell types absent from a specific culture condition. After an additional 12 to 24 hours, the response of each cell type will be characterized by assaying for general changes in morphology, neuronal death, and, when appropriate, microglia activation or glial proliferation according to standard protocols (as described above).

Possible Outcomes and Problems. If PrP^{SC} interacts with a specific cell type causing release of a diffusible factor (or factors) that are sufficient to mediate neuronal toxicity, then conditioned media from that PrP^{SC}-treated cell-type specific culture (from the first set of experiments) will cause neuronal toxicity in the neuron-only cultures (from the second set of experiments). If this diffusible factor requires another cell type in order to mediate neuronal toxicity, only neurons grown with that additional cell type will show signs of neuronal toxicity. For example, if PrP^{SC} treatment causes microglial release of a diffusible factor that is sufficient to induce neuronal toxicity, then conditioned media from PrP^{SC}-treated microglia-only cultures will cause neuronal toxicity in neuron-only cultures. If, however, PrP^{SC}-induced microglial causes release of a diffusible factor that, in turn, causes astrocytic release of a diffusible factor that is sufficient to induce neuronal toxicity, then conditioned media from PrP^{SC}-treated microglia-only cultures will cause neuronal toxicity only in cultures containing both neurons and astrocytes.

These experiments require neuron-only cultures, which have proven difficult in the past. However, based on work by Ullian *et al.*, who were able to culture retinal ganglion cells in the presence of astrocyte-conditioned media, we believe that astrocyte- and microglia-conditioned media will be sufficient to sustain cortical neurons in culture (2001). If this does not work, Banker cultures or a modified culture protocol could be used to prevent direct contact between neurons and astrocytes (Banker and Goslin, 1998). The second set of experiments may show that conditioned media from PrP^{SC}-treated cell-type specific cultures is insufficient to cause neuronal toxicity. One explanation of these findings would be that PrP^{SC} forms a complex with the diffusible factor that is then retained by the affinity column. To verify this possibility, the complex would be eluted from the column and run on a Western blot to check for a molecular weight larger than that expected for PrP^{SC} alone. Alternatively, a diffusible factor may not be responsible for mediating neuronal toxicity and/or neuronal toxicity may require cell-cell contact. Using conditioned media from PrP^{SC}-treated pairs of cell types would begin to address these questions.

Future Directions. If these experiments prove successful, future experiments will use a similar experimental design to study the effects of PrP^{SC}-induced toxicity on combinations of WT and $Prnp^{0/0}$ neurons, astrocytes, and microglia in an effort to determine which cell types must express PrP^{C} in order to mediate toxicity. Future experiments will also aimed at determining if the peptide fragment PrP106-126 has the same *in vitro* effects as the full-length protein PrP^{SC}.

Aim 2 (a) *Generate transgenic mouse with inducible ablation of microglia, monocytes, and macrophages and characterize mouse phenotype after loss of these cell types.*

Experimental Design. In order to determine whether microglia play a role in prion disease pathology, we wish to generate a mouse lacking microglia as an *in vivo* model. We hypothesize that the role of microglia during adulthood lies primarily in its function during disease states. Thus, we would like to characterize the phenotype of an adult mouse with microglial ablation. Because microglia are thought to play an important role during neural development, ablation in our model will have to be inducible after birth. An inducible ablation of these cells in adult mice avoids disease-independent developmental effects and is desirable in future aims to allow examination of microglial contribution during different phases of disease. We also seek to use ablation methods that relatively simple mouse genetics to increase our probability of success.

To this end, we will use an inducible ablation method as recently described by Saito et al (2001.) In species susceptible to diphtheria toxin (DT), DT enters the cell by binding to the heparin-binding EGF-like precursor (HB-EGF). Cell ablation in this method depends on the fact that mice and rats express a form of HB-EGF that does not recognize DT, rendering them immune to high levels of toxin. However, expression of the

human HB-EGF in mouse hepatocytes has been shown to confer susceptibility in those cells to DT. Thus, transgenic mice that drive expression of HB-EGF through a cell-type specific promoter should cause cell-type specific death after administration of DT. Unlike other ablation methods, creation of these mice does not require homologous recombination or crossing of transgenic strains, and mice can be analyzed from the F1 generation.

This method requires a cell-specific promoter. We will use the promoter from F4/80, a cell surface receptor expressed specifically by monocytes, macrophages, and microglia (Carson et al, 1998, McKnight et al, 1996). Because it is unclear whether new microglia can be formed in the adult animal from circulating macrophages and monocytes, ablation of all cells expressing F4/80 should eliminate all microglia in the brain. We will first define the F4/80+ promoter region necessary for cell type-specific transcription by promoter trapping experiments in a monocyte cell line as was performed for another monocyte marker by Yamamoto, et al (1999). Once the promoter region is identified, we will construct a transgene with F4/80 promoter upstream of HB-EGF cDNA. To create the mice, the protocol as described in Saito et al (2001) will be used. Briefly, the DNA transgene will be microinjected into fertilized mouse eggs, which will then be implanted into pseudopregnant female mice. Integration of transgene into the genomic DNA of progeny will be assessed by PCR analysis. We will look in several lines for high levels of HB-EGF RNA transcripts in macrophages and microglia, which can be purified by FACS using F4/80 or other macrophage/monocyte/microglial markers. We will then confirm cell specificity with northern blot analysis and *in situ* hybridization in various cell types and tissues, including neurons and astrocytes.

Ectopic expression of the receptor has been shown to have no phenotype by Saito et al. However, we will need to confirm there is no deleterious phenotype in these mice by examining longevity and general phenotype of uninduced transgenic mice. We will then test for specific cell ablation after administration of DT at dosages shown to be harmless in wild type mice. Successful ablation will be assessed by decrease in macrophage/monocyte/microglia numbers and signs of cell death in macrophages, monocytes, and microglia (as determined by FACS). Histology should reveal an absence of microglia in brains of DT treated mice. Because Saito et al showed the effects of DT wear off within ~150 hours, DT treatment for complete ablation of microglia, monocytes, and macrophages will most likely need to be chronic. DT dosage levels and administration routes will be assessed for optimal chronic ablation of microglia, monocytes, and macrophages. We will need to assess the general phenotype of chronic DT-treated wild type mice. We will especially observe longevity and general health of DT-treated transgenic mice as compared to untreated transgenic controls. Scoring will include brain histology.

Possible Outcomes and Problems. We expect mice to show little or no phenotype after administration of DT. A phenotype due to macrophage/monocyte ablation is unexpected because it is known that transgenic mice with severe defects in macrophage/monocyte function develop normally and are otherwise healthy if their immune systems are not challenged. Microglial function during adulthood is not fully understood, but current evidence suggests they are quiescent until activated in diseased states (Barron, 1995). However, if there is a pathological effect of microglial ablation in the brain, this would be extremely informative about the currently uncharacterized functions of microglia in normal brain homeostasis and should be investigated further. These effects may not compromise experiments proposed in aim 2 if using lower doses of DT to reduce numbers of microglia without complete ablation can alleviate such a phenotype. This also means we would be scoring for the effect of partial phenotypes.

We may experience difficulties in generating mice, or HB-EGF may have unexpected deleterious side effects when expressed in these cell types. Furthermore, although we expect chronic treatment of DT to be harmless in HB-EGF -/- cells, this has not been assessed. In any of these cases, inducible ablation through transgenic mice expressing the herpes thymidine kinase under the F4/80 promoter is an alternate strategy. This kinase has been shown to be harmless in cells, including glial cells, but can cause toxic upon application of normally innocuous nucleoside analogs acyclovir and FIAU. Vandier et al were able to induce astrocyte-specific ablation. If the F4/80 promoter region is too large or cannot be isolated, we can try promoter regions from other macrophage/monocyte/microglial markers such as Mac1.

Future Directions. Besides being an important model system for examining the role of microglia during prion disease pathogenesis, this mouse would be useful for many other directions of research. We could further

examine the normal role of microglia, macrophages, and monocytes during development or during non-neurological immune challenges. Because microglia are thought to be important in both inflammatory processes in the brain and the pathogenesis of HIV and Alzheimer's, this mouse model could also be readily applied to other diseases (McGeer & McGeer, 1998; Adle-Biassette, *et al*., 1999).

Aim 2. (b) *Determine whether microglia are necessary for prion disease pathogenesis in vivo.*

Experimental Design: Production of mice conditionally lacking microglia, macrophages, and monocytes in Aim 2a will allow us to analyze the role of microglia in prion disease. Transgenic mice and their wild type littermates will be intracranially injected with PrP^{SC}-containing brain homogenates, as in Prusiner, *et al.* (1993). In the first experiment, microglia will be knocked out with DT, as described in Aim 2a, prior to PrP^{SC} injection. The mice will be followed to determine the course of disease and establish time points for histological analysis. In the second experiment, microglia ablation will start at the following time points: before exposure to PrP^{SC}, after exposure to PrP^{SC} but before the onset of symptoms, and after the onset of symptoms. As controls, transgenic and wild type mice will be injected with PrP^{SC}-containing brain homogenates then saline or with brain homogenates from wild type animals followed by DT. Following injection, the mice will be regularly scored for signs of neurological dysfunction. Mice will be sacrificed at three time points: before the onset of symptoms, in the middle of the symptomatic stage, and during end stage disease. Mice showing no signs of disease will be sacrificed after 500 days. PrP^{SC} levels in the brains of the mice will be measured using *in situ* immunoblotting; histological scoring of gliosis, plaque-formation, and vacuolization will be performed as in Brandner, *et al.*, (1996).

Possible Outcomes and Problems. Increased microglial numbers have been shown to increase neurotoxicity in culture in combination with the PrP106-126 protein fragment (Brown, *et al.*, 1996). Therefore we expect to see a decrease in neurotoxicity in mice with microglial ablation, resulting in an increased time to symptom onset and/or an increased time to death. Microglial removal should not change the proliferation of PrP^{SC}, but may reduce the number neurons entering apoptosis, if the release of toxic compounds by microglia is key to the neurodegeneration. Knockouts made during the symptomatic period of the disease are expected to reduce further degeneration and gliosis. Prion disease should be induced in wild type controls in approximately 150 days.

No change in the course of the induced prion disease in the knockout mice would indicate either that PrP^{SC} has toxic effects independent of the presence of microglia, or that positive and negative impacts of microglia on the disease balance out. It is also possible that the ablation of microglia will increase the pathogenesis of the disease, indicating an important role for microglia in neurotrophic support during disease.

This model system will be useful for further studies of the role of microglia in prion diseases and other neurodegenerative disorders. Future studies may be able to selectively ablate microglia or macrophages, allowing investigation into the role of the resident microglia in establishing local inflammation in the brain, but also of the peripheral macrophages in neuroinvasion by prions. Finally, this system could be used to begin to investigate potential treatments of prion diseases that involved reduction of the activation of microglia.

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