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Letter to Neuroscience

EVIDENCE FOR AN EARLY INFLAMMATORY RESPONSE IN THE CENTRAL NERVOUS SYSTEM OF MICE WITH SCRAPIE

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In Alzheimer's disease, the most prevalent of the neurodegenerative diseases, inflammation of the CNS contributes to the pathology and is a target for therapy.[13,18,19] In contrast, the group of neurodegenerative conditions known as the Prion Diseases have been widely reported as lacking any inflammatory elements[5,23] despite the many similarities between the pathologies of Alzheimer's Disease and Prion Diseases.[10] We have found evidence for an inflammatory component in mouse scrapie, characterized by microglial activation and T-lymphocyte recruitment, which appears long before any clinical signs of the disease and spreads along well-defined anatomical pathways. These observations emphasize the potential value of murine scrapie as a model for studying the inflammatory pathology of other neurodegenerative diseases. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Prion diseases (PD), also known as Transmissible Spongiform Encephalopathies, are diseases of humans (e.g., Creutzfeldt Jacob disease) and animals (e.g., Scrapie of sheep and Bovine Spongiform Encephalopathy of cattle). Scrapie can be transmitted experimentally to laboratory rodents by injection of scrapie infected brain homogenate.[7] PD typically have a long incubation period and are characterized neuropathologically by gliosis, neuronal loss and vacuolation. They can occur sporadically or have a genetic or infective aetiology.[9] The causative agent is believed to be an abnormal isoform of a normal cellular prion protein, PrP^{c}, which is modified by an unknown mechanism to become an infectious agent, the proteinase-resistant prion protein, PrP^{sc}.[22] The links between the PrP^{sc} deposits and neurodegeneration are not established although PrP^{sc} has been shown to be neurotoxic *in vitro*.[14]

Several authors have commented on similarities between PD and Alzheimer's Disease (AD) pathology,[10] but although CNS inflammation is present in AD[18] and anti-inflammatory therapy appears to influence the course of the disease,[4,25] it is surprising that PD are said to lack any inflammatory or immune component.[5,23] Inflammation in the CNS has a very different appearance to that in peripheral sites[21] and the techniques required to investigate it are not those used traditionally. Using experimental approaches of proven value in the study of CNS inflammation[21] we have investigated the putative inflammatory component in mouse scrapie.

Clinical signs typical of scrapie including hunched posture, ruffling of fur and reduction of mobility[7] were first observed by 23 weeks after intracerebral injection with ME7 (original source: Suffolk sheep natural Scrapie) infected brain homogenate. Terminal disease occurred between 24 and 27 weeks post-injection ($n = 10$). Control animals injected with normal brain homogenate did not develop any clinical signs and have been disease-free for 12–18 months post injection ($n = 6$).

Examination of the brains of ME7-injected mice revealed widespread and bilateral prominent astrocytosis, neuronal loss and microglial activation, consistent with previous descriptions of prion-infected mouse brains[7,29] (Fig. 1a–c). The activated microglia had many short processes and expressed high levels of macrosialin and MHC Class I when compared to microglia in normal brain. The regions affected included the hippocampus and thalamus bilaterally, the septum, pons and spinal cord.

The earliest evidence of pathology in ME7-injected animals was seen eight weeks post-injection with

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Abbreviations: AD, Alzheimer's Disease; GFAP, glial fibrillary acidic protein; MHC, Major Histocompatibility Complex; PD, Prion diseases; PrP^{c}, normal, cellular prion protein; PrP^{Sc}, disease-associated, proteinase resistant prion protein; ME7, Scrapie strain inoculum.

hypertrophied glial fibrillary acidic protein-positive (GFAP+) astrocytes and activated microglia around the injection site in the dorsal hippocampus ($n = 3$). In addition, there was a small number of rounded cells which expressed the pan lymphocyte antigen CD3. On adjacent sections stained with anti-CD4 or anti-CD8 antibodies most of the lymphocytes were of the CD8+ phenotype with a few CD4+ cells present. The lymphocytes, microglia and the endothelium expressed MHC Class I antigens. At this time there was no evidence of neuronal degeneration in the CA fields of the hippocampus or in the dorsal thalamus.

Lymphocytes always appeared first at the locus of the intra-cranial injection: for example, in animals injected into the brainstem ($n = 2$) or the hypothalamus ($n = 2$) and examined at 12 weeks post-injection there were more T-lymphocytes in the region of the injection site than other sites such as the dorsal hippocampus.

From eight weeks onwards, there followed distinct, progressive stages in the development of the pathology with astrocyte hypertrophy and enhanced GFAP expression, microglia activation and T-lymphocyte recruitment. At 12 weeks post-injection ($n = 8$) there was microglial activation involving the whole of the dorsal region of the injected hippocampus and some involvement of the uninjected hippocampus, the septum, diagonal band and dorsolateral nuclei of the thalamus on the injected side (Fig. 1d,f,g). The pathology in the uninjected hippocampus, septum and diagonal band is consistent with spread along known anatomical pathways,[26] but the pathology in the dorsal and lateral thalamus may result from diffusion of the pathological agent since there are no known connections between the lateral thalamic nucleus and the hippocampus. By 16 weeks post-injection ($n = 7$) there was pronounced bilateral hippocampal and increased thalamic involvement, the latter becoming further increased by 20 weeks post-injection ($n = 8$). When clinical signs first appeared at 23 weeks post injection ($n = 8$), there was clear evidence of microglia activation and T-cell infiltration into the cortex overlying the hippocampus and also other cortical areas. There was about a 10-fold increase in the number of microglia in the dorsal hippocampus of ME7 injected animals compared with controls at 23 weeks post-injection. The systematic spread of the pathology at the coronal level of the injection site is illustrated in Fig. 2 by the distribution of CD8+ T-lymphocytes at different times post injection. The number of CD8+ cells always outnumbered the CD4+ cells; for example, at 23 weeks post-injection 92% of total lymphocytes were CD8+. Examination of sections along the rostrocaudal axis of the brain from the septum to the brainstem, and additional sections from the cervical and lumbar levels of the spinal cord in terminal disease, revealed that the spread of the astrocytic response was in all cases associated with microglial activation and T-lymphocyte recruitment.

Fig. 1. Alcohol fixed 10 μm cryostat sections showing cresyl staining of CA1 region in (a) normal brain homogenate injected C57BL/6J mice and (b) ME7 injected mice at terminal disease. Note the reduced thickness of the CA1 cell layer and increased cellularity of the surrounding parenchyma in the section from a terminally affected animal. The astrocytosis characteristic of the prion diseases is illustrated in (c) with GFAP immunocytochemistry. Consecutive sections from the region of the dorsal thalamic nuclei at 12 weeks post-injection with ME7 showing: (d) FA11 immunocytochemsitry showing microglial activation, (f) the presence of CD3+ cells which are further characterised as (g) CD8+ cells. Scale bar = 50 μm. Male and female two- to three-month-old C57BL/6J mice were bred and housed in the Department of Pharmacology Mouse Unit under standard laboratory conditions with free access to food and water. The animals were anaesthetised with i.p. Avertin (0.1 ml/5 g weight) and held in a stereotaxic frame. One microlitre of 10% w/v of ME7-infected C57BL/6J brain homogenate in normal saline was injected into the right dorsal hippocampus ($n = 44$ total); bregma -2.5 mm, lateral -1.7 mm, depth -1.6 mm via a pulled glass micropipette. Control animals were also injected stereotactically into the right hippocampus with 1 μl of 10% w/v of normal C57BL/6J brain homogenate in 0.9% saline ($n = 28$ total). Additional animals were injected with 1 μl of 10% ME7 into the brainstem ($n = 2$) and the hypothalamus ($n = 2$). A random sample of animals housed in the mouse unit underwent a comprehensive infection screen for viruses and bacteria which was negative. We used 10% w/v brain homogenates derived from two separate batches of ME7 scrapie-infected mice, and one batch of 22L scrapie infected animals. These homogenates were from animals maintainted in the SPF unit of the AFRC, Edinburgh. A 10% w/v brain homogenate of normal brain was derived from the same source and an additional normal brain homogenate was prepared in the Department of Pharmacology Mouse Unit. All injected animals were housed in a Scantainer Type D (Scanbur Ltd, Denmark) and observed regularly. Animals were perfused at 4 weekly intervals starting at eight weeks post-injection until the appearance of clinical signs. Animals were deeply anaesthetised and trans-cardiac perfusion with 0.9% heparinised saline was performed. The brains were embedded in Tissue Tec (OCT embedding compound, Miles Inc.) and frozen in isopentane on liquid nitrogen. Cryostat sections from fresh-frozen brains were fixed in absolute alcohol for 10 min at 4°C. In all groups immunocytochemistry was carried out on coronal 10-μm cryostat sections, using primary antibodies as follows: FA11 to detect macrosialin (CD68), a lysosomal antigen in macrophages;[24] KT3 to detect CD3, a pan T-lymphocyte antigen;[28] YTS191 and YTS169 to detect CD4+ and CD8+ T-lymphocytes respectively;[8] M1/42 to detect MHC class I;[27] and an antiserum to glial fibrillary acidic protein (GFAP) to detect astrocytes (DAKO). The primary antibodies were detected by the avidin–biotin peroxidase method using reagents from Vector Laboratories (Vesctastatin Elite ABC) and diaminobenzidine as the chromogen as previously described.[15]

The presence of T-lymphocytes and activated microglia in animals with scrapie was not associated with the surgery or the injection of brain homogenate *per se* since they were not present in the brain parenchyma of animals injected with normal brain homogenate derived from a Specific Pathogen Free (SPF) unit ($n = 4$), or from our own animal unit ($n = 17$). The presence of activated microglia and T-lymphocytes was present after injection with two separate batches of ME7. Early microglial activation and T-lymphocyte recruitment was not just a peculiarity of the ME7 scrapie strain since both classes of cells were also seen in animals injected with the 22L strain.

The current received wisdom that PD lack an inflammatory element (at least in the pre-terminal stages of the disease) has probably arisen because: (i) inflammation in the CNS is different in several respects from peripheral inflammation;[21] (ii) the reagents for studying it are less widely available; (iii) the preparation of the tissue needs to be approached differently. We have used fresh frozen cryostat sections rather than paraffin sections, and this could be why we have been able to detect CD8+ T-cells. Immunocytochemistry using the antibody YTS169 on 2% paraformaldehyde perfused tissue produced inconsistent CD8+ T-cell staining, while fixation of fresh frozen cryostat sections with either 2%

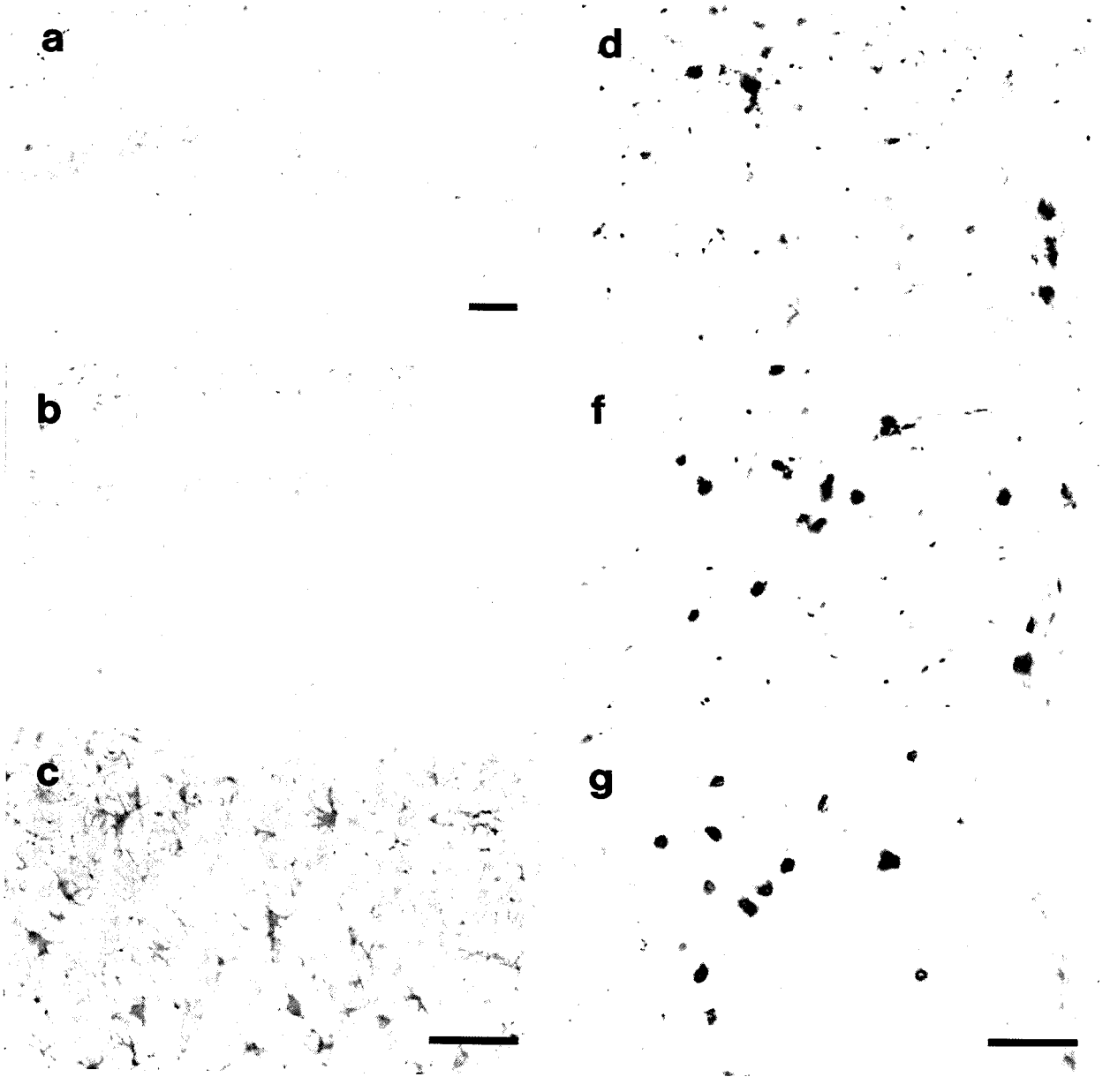

Fig. 1.

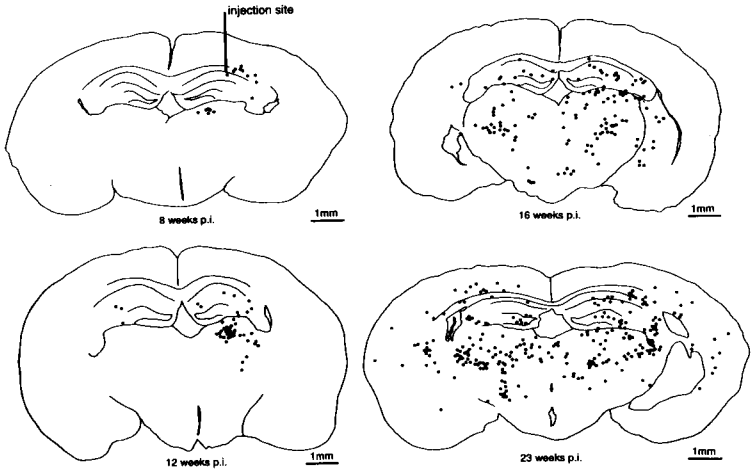

Fig. 2. Diagram illustrating the progress of CD8+ lymphocyte distribution at the different survival times indicated following focal injection of ME7. The representative coronal sections at the level of the injection site were drawn using a microscope drawing tube. Each drawing shows the distribution in a single 10-μm section, each spot represents the location of one CD8+ lymphocyte.

paraformaldehyde or 0.2% gluteraldehyde completely abolished staining for CD8+ T-cells. Therefore the method of tissue preparation and fixation is critical for CD8+ immunocytochemistry. It should be noted that inflammation has been seen in terminal PD; indeed, given the extent of tissue damage seen in the final stages of the disease, this is not surprising. These previous findings[29] did not however provide any hints that inflammation early in the course of the disease might contribute to pathology.

The findings reported here do not unequivocally prove whether the inflammation in PD is contributory or consequential, but its appearance so early in the course of the disease suggests the former explanation. Other evidence also points in this direction: firstly, co-infection of mice with a non-pathogenic adenovirus at various times after inoculation with scrapie leads to faster progression of the disease;[11] secondly, treatment of mice with anti-inflammatory steroids delays the clinical signs of disease progression.[20] Whether the T-lymphocytes and the activated microglia/monocytes both contribute to the pathology remains to be determined. Although nude mice (with very few functional T cells) can develop scrapie[17] the fact that CD8+ cells appear in association with the astrocytosis and microglial activation in a well-defined spatial and temporal pattern suggests that the function of the T-cells is to monitor neurodegeneration. It should be noted that although the blood-brain barrier appears to be intact even in the terminal stages of scrapie[12] this does not prevent monocytes from entering the brain.[2]

The findings we report here could have significant implications for future research on other neurodegenerative diseases (notably AD). Although there are many differences between AD and PD, there are also parallels. For example, both involve aberrations of the processing of normal neuronal plasma membrane proteins[6,30] the abnormal proteins accumulate as insoluble deposits in the form of beta-pleated sheets[10] and are neurotoxic,[14,16] the disease spreads in a predictable pattern along anatomical pathways.[3,26] Consequently, further investigation of the inflammatory pathogenesis of PD may provide insights into the pathogenesis of AD. At the very least, our findings indicate that mouse scrapie can provide a valuable model for investigating how the inflammatory response contributes to the pathology of chronic neurodegenerative disease.

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