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Scrapie Agent Proteins Do Not Accumulate in Grey Tremor Mice

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SUMMARY

The grey tremor mouse is an autosomal recessive mutant characterized by a phenotype of unusual pigmentation, neurological abnormalities and early death. These mice have a spongiform encephalopathy similar to scrapie and Creutzfeldt-Jakob disease. Although the disease is clearly heritable, the grey tremor mouse spongiform pathology has also been transmitted by inoculation of genetically normal mice with diseased brain homogenates. The possibility that a scrapie-like agent is involved has been proposed. We examined brain homogenates from grey tremor mice, scrapie-affected mice and normal mice for the presence of the mouse scrapie agent protein (MoSp33–37) and its normal cellular homologue. All untreated homogenates contained one or both isoforms of this protein as detected on immunoblots. Grey tremor mouse brain homogenates, when protease-treated, showed no evidence of MoSp33–37. A purification method for MoSp33–37 concentrated it in samples from scrapie-affected mice, but this protein was not detected in grey tremor or normal mice. These results suggest that it is unlikely that the scrapie agent is involved in grey tremor disease.

Mice homozygous for the grey tremor (GT) trait (gt/gt) display a characteristic pigmentation of their coats, seizures, tremors, ataxia and death by 90 days of age (Sidman *et al.*, 1985). The neuropathology is a non-inflammatory spongiform appearance to the brain (Kinney & Sidman, 1986) resembling that of the transmissible spongiform diseases scrapie and Creutzfeldt-Jakob disease (CJD). The spongiform neuropathology, but not the neurological syndrome characteristic of the homozygote gt/gt, was transmitted to genetically normal mice by inoculation with gt/gt brain homogenates (Sidman *et al.*, 1985). In a second report on transmission of GT disease, NFS/N mice were inoculated with gt/gt brain homogenate (Hoffman *et al.*, 1987). Sixty days later they started to display a syndrome of tremulousness that progressed over 300 to 400 days to a marked ataxia, spasticity and bradykinesia. This transmitted syndrome was different to that which occurred in gt/gt homozygotes and differed from the syndromes which appeared in NFS/N mice inoculated with either a murine leukaemia virus or with the scrapie agent.

Mouse scrapie is a stereotypic clinical and neuropathological disease experimentally reproduced by intracerebral inoculation of the scrapie agent into animals. In C57BL mice inoculated with the ME7 strain of the scrapie agent a silent incubation period of 120 to 180 days is followed by a uniformly fatal neurological syndrome lasting 3 to 4 weeks. The major signs are a hunched posture, ataxia, tremulousness and preterminal immobility. The disease course and clinical signs of scrapie in mice are distinct from those of GT disease.

The physicochemical nature of the agents responsible for scrapie in animals and CJD, kuru and the Gerstmann-Straussler syndrome in humans is not completely defined (reviewed by Carp *et al.*, 1985; Diener, 1987). However, in all natural and experimental hosts examined, unique, scrapie-specific proteins have been detected (Bolton *et al.*, 1982, 1984; Bendheim *et al.*, 1985; Bockman *et al.*, 1987; Rubenstein *et al.*, 1987). In each species these proteins are derived from a

Fig. 1. Identification of MoCp33-37 and MoSp33-37 by immunoblotting. Brain homogenates (10% w/v) were made from single brains in 0.32 M-sucrose using a glass-Teflon homogenizer. An aliquot of each homogenate was diluted with distilled water and SDS-PAGE sample buffer (62.5 mM-Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.0002% bromophenol blue, pH 6.8) to a final concentration of 25 mg/ml. Samples from unaffected (+/gt or +/+) GT littermates (lane 1), symptomatic GT homozygotes (gt/gt) (lane 2), normal C57BL mice (lane 3) and scrapie-affected C57BL mice (lane 4) were subjected to SDS-PAGE and analysed by immunoblotting using an antiserum generated against PrP27-30, a protease-resistant fragment of the scrapie protein (Bendheim *et al.*, 1984). Molecular size markers are indicated.

single normal host gene encoding a cellular protein of unknown function (Oesch *et al.*, 1985; Basler *et al.*, 1986; Lochter *et al.*, 1986; Robakis *et al.*, 1986). The abnormal form of this protein is only detected in scrapie- and CJD-diseased brains and its amount correlates with the amount of biologically active agent (McKinley *et al.*, 1983; Brown *et al.*, 1986; Bolton *et al.*, 1987). This and other experimental data implicate this protein as the major structural protein of the transmissible agent. In the literature the terms 'scrapie agent protein', 'protease-resistant protein' and 'prion protein' have been used in reference to those proteins. In this report we use the term scrapie agent protein (Sp33-37) for the intact protein and protease-resistant protein (PrP) for the truncated form produced by protease digestion of the intact protein (Bolton *et al.*, 1987).

We searched for scrapie agent-specific proteins in GT mouse brain to investigate further the suggestion by Sidman *et al.* (1985) that the scrapie agent might be involved in the transmission of the spongiform encephalopathy characteristic of this mutant.

Mice were purchased from Jackson Laboratories (Bar Harbor, Me., U.S.A.). All gt/gt mice displayed the coat colour and marked neurological impairment characteristic of this strain. Littermates of the gt/gt homozygotes were phenotypically normal +/gt heterozygotes or +/+ homozygotes. C57BL mice inoculated intracerebrally with scrapie agent strain ME7 served as scrapie-positive controls and uninoculated age-matched C57BL mice were normal controls. Grey tremor mice and their littermates were sacrificed at 50 to 72 days of age, scrapie-affected C57BL mice at 120 to 140 days post-inoculation (165 to 220 days of age) and normal C57BL mice at 165 to 220 days of age.

Fig. 1 is an immunoblot of proteins in brain homogenates of phenotypically normal GT littermates (either +/gt or +/+) (lane 1), symptomatic GT gt/gt mutants (lane 2), normal C57BL mice (lane 3) and scrapie-affected C57BL mice (lane 4). The antiserum used was raised against the hamster scrapie agent protein (Bendheim *et al.*, 1984) and has been shown to identify the major mouse scrapie agent proteins (Bendheim & Bolton, 1986). It also recognizes the cellular protein (Cp33-37) which is the normal translation product of the gene encoding both isoforms of this protein. Immunoreactive proteins of M_r 33K to 37K were detected in all four samples. In the scrapie agent-containing sample (lane 4) darker staining is evident, as well as staining of several additional lower M_r proteins. This pattern of immunoreactivity suggests the presence of increased amounts of mouse scrapie agent protein (MoSp33-37) or mouse cellular protein (MoCp33-37) when compared with lanes 1 to 3. That proteins were identified in the

Fig. 2.

Fig. 3.

Fig. 2 Protease digestion of proteins in GT and scrapie-affected mouse brains. Aliquots of the homogenates shown in Fig. 1, lanes 2 and 4, were incubated for 30 min at 37 °C with 50 µg/ml proteinase K (EM Reagents, F.R.G.). The reaction was stopped by the addition of PMSF (Sigma, final concentration 20 mM), SDS-PAGE sample buffer and heating at 100 °C for 5 min prior to SDS-PAGE and immunoblotting as described in Fig. 1. Lane 1, GT homozygote; lane 2, scrapie-affected C57BL. The scrapie agent-specific protein bands present in lane 2 are absent from lane 1. The arrow indicates that in both lanes proteinase K was recognized by this antiserum (Bendheim *et al.*, 1984).

Fig. 3. Purification of MoSp33-37 is possible from scrapie-affected mouse brain, but not from GT mouse brain. Two brains each from C57BL mice with scrapie and GT mice were processed using a modification of a method developed to purify the scrapie agent protein from hamster brain (Bolton *et al.*, 1987). Pellets (P_4) so prepared were resuspended in nuclease digestion buffer (10 mM-Tris-HCl, 100 mM-NaCl, 5 mM-CaCl₂, 5 mM-MgCl₂, pH 7.4). RNase A (100 µg/ml) and DNase I (20 µg/ml) were added and the suspensions incubated for 2 h at room temperature with continuous stirring. EDTA, NaCl and Sarkosyl were added to final concentrations of 50 mM, 10% (w/v) and 1%, respectively, and the samples were sedimented in a microcentrifuge for 30 min at room temperature. The pellets (P_5) from a GT gt/gt preparation (lane 1) and a scrapie-affected C57BL preparation (lane 2) were analysed on an immunoblot as described in Fig. 1.

diseased gt/gt mice and their normal littermates (lanes 1 and 2) demonstrated that the brains of these mice contained one or both isoforms of the 33K to 37K protein. However, these results could not distinguish between the two isoforms.

When brain homogenates are treated with proteinase K Cp33-37 is completely degraded (Barry *et al.*, 1985). This criterion of differential protease sensitivity allows the distinction between the two isoforms of the protein to be made. In an attempt to determine whether MoSp33-37 was present at low concentration in GT mice, but obscured by MoCp33-37, a sample of the GT brain homogenate was treated with proteinase K. An aliquot of the scrapie-affected C57BL mouse brain homogenate was used as a control. In the GT sample no degradation-resistant scrapie agent proteins were detected (Fig. 2, lane 1). Scrapie agent-specific PrPs were detected in the scrapie-affected C57BL mouse sample (Fig. 2, lane 2). These proteins have apparent M_r s of 28K to 31K, 23K to 26K and 21K and are similar to those

Table 1. *Features of grey tremor and scrapie in mice*

	GT	Scrapie
Heritable	+	—
Transmissible pathology	+	+
Transmissible clinical disease	?	+
Cp33–37	+	+
Sp33–37	—	+
Scrapie-associated fibrils	—	+
Chromosomal localization†	15	2

* +, Present; —, absent.

† The gt mutation has been linked to the caracul locus on chromosome 15 (Sidman *et al.*, 1985). The gene(s) for scrapie incubation period control and encoding Cp33–37 and Sp33–37 are on chromosome 2 (Sparkes *et al.*, 1986; Carlson *et al.*, 1986).

described previously (Bolton *et al.*, 1984; Kascsak *et al.*, 1986). The staining of proteinase K by the antiserum used in this experiment (Fig. 2, lanes 1 and 2, arrow) has also been noted before (Bendheim *et al.*, 1984). These results demonstrated that no protease-resistant scrapie agent proteins were detected in GT brain and indicated that MoCp33–37 was predominant, if not the only, isoform of the protein that had been detected in the homogenate of GT brain (Fig. 1, lane 2).

It was still possible that MoSp33–37 was present in GT brain homogenates at a concentration below that required for detection on immunoblots. Therefore, an attempt was made to purify and concentrate this protein from GT mouse brains. Cp33–37 and Sp33–37 can be separated on the basis of their different physical properties by a method previously described for the purification of the intact scrapie agent protein from diseased hamster brains (Bolton *et al.*, 1987). As demonstrated in Fig. 1, an isoform of the protein was present in the initial GT brain homogenate. However, scrapie agent proteins were not detected in the P₅ fraction from GT mouse brain (Fig. 3, lane 1). MoSp33–37 and several lower *M_r* antigenically related proteins were readily detected in the P₅ fraction from scrapie-affected C57BL mice (Fig. 3, lane 2), demonstrating that scrapie agent proteins were concentrated in this fraction. The lower *M_r* proteins probably represent degradation products of MoSp33–37 or incompletely processed forms of the primary translation product. These results demonstrated that MoSp33–37 was not purified from gt/gt brains using this procedure for isolating the scrapie agent.

The cardinal features of GT disease and scrapie are summarized in Table 1. The pathological features of the spongiform encephalopathy of the GT mutant resembles those of scrapie, although clinical features of the two diseases differ. Scrapie is transmissible in its entirety: host animals develop the characteristic clinical syndrome and neuropathology. Previous work on the GT mutant demonstrated that the pathological changes characteristic of the homozygote could be transmitted to genotypically normal mice after an incubation period as long as 2 years (Sidman *et al.*, 1985). Mice inoculated with gt/gt brain homogenates did not develop a clinical syndrome resembling that of either the gt/gt homozygote or scrapie (Sidman *et al.*, 1985; Hoffman *et al.*, 1987). Some mice appeared abnormal after inoculation, but when progression of clinical disease occurred it was milder and slower than in the gt/gt mutant. This is in marked contradistinction to scrapie-affected mice, all of which demonstrate a relentless progression of neurological signs leading to death within weeks after the onset of disease.

The scrapie protein, Sp33–37, accumulates in all animals with scrapie and similar proteins accumulate in humans and experimental animals with CJD. It is a modified form of a normal, host-derived protein. The two forms of the protein differ in certain physical properties and have been distinguished and separated on this basis (Meyer *et al.*, 1986; Hope *et al.*, 1986; Bolton *et al.*, 1987). Our results demonstrate that GT mice contain only the normal isoform. It appears to be present in quantities similar to those in normal mice of other strains. No evidence for the presence of the scrapie agent protein was found in either GT brain homogenates treated with proteinase K or in P₅ fractions from a purification protocol used to isolate the abnormal protein and the biologically active scrapie agent.

The absence of the scrapie isoform of the protein argues against the scrapie agent being involved in the transmission of the GT pathology. These results are consistent with those of others. Scrapie-associated fibrils have been detected in all the natural or experimental animal and human transmissible spongiform encephalopathies caused by scrapie or CJD agents (Merz *et al.*, 1984). These characteristic fibrils have not been detected in GT brains (Sidman *et al.*, 1985; Hoffman *et al.*, 1987). The single gene encoding both MoSp33-37 and MoCp33-37 is on chromosome 2 in the mouse (Sparkes *et al.*, 1986). This gene is closely linked to, and may be identical with, the primary gene controlling scrapie incubation period duration (Carlson *et al.*, 1986). The GT mutation is linked to chromosome 15 (Sidman *et al.*, 1985). Therefore, the agent responsible for experimental transmission of the GT spongiform neuropathology remains to be identified, but these data indicate that the scrapie agent is not the pathogen.

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