

Metal imbalance and compromised antioxidant function are early changes in prion disease

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The prion protein (PrP) has been shown to bind copper. In the present study we have investigated whether prion disease in a mouse scrapie model resulted in modification of metal concentrations. We found changes in the levels of copper and manganese in the brains of scrapie-infected mice prior to the onset of clinical symptoms. Interestingly, we noted a major increase in blood manganese in the early stages of disease. Analysis of purified PrP from the brains of scrapie-infected mice also showed a reduction

in copper binding to the protein and a proportional decrease in antioxidant activity between 30 and 60 days post-inoculation. We postulate that alterations in trace-element metabolism as a result of changes in metal binding to PrP are central to the pathological modifications in prion disease.

Key words: copper, manganese, neurodegeneration, scrapie, superoxide.

INTRODUCTION

Prion diseases are fatal neurodegenerative disorders in which neuronal loss and gliosis occur after a long, apparently asymptomatic incubation period [1]. These diseases include bovine spongiform encephalopathy ('BSE') [2], scrapie [3] and Creutzfeldt–Jakob disease ('CJD') [4]. The causative agent in these diseases is believed to be an abnormal disease-specific conformation (PrP^{Sc}) [1] of the normal cellular prion protein (PrP^C). It has been shown that PrP^{Sc} accumulates before the onset of symptoms and neuronal death [5]. Genetically modified mice that lack expression of PrP^C are resistant to infection with mouse prion inoculum [6]. Therefore it appears that expression of PrP^C is necessary for prion disease. Understanding the differences between PrP^C and PrP^{Sc} is central to developing a mechanism for how this conformational change leads to neurodegeneration.

PrP^C has been shown to be a copper-binding protein [7–9] and may bind approx. 4 atoms of copper per molecule, via its octameric repeat region. Native PrP^C purified from mouse brains has on average 3 atoms of copper per molecule [10]. It has been shown that both purified native and recombinant PrP^C possess a catalytic activity similar to that of superoxide dismutase (SOD) which is dependent on the binding of copper [10,11]. PrP^C is a synaptic protein and depletion of PrP^C from synapses leads to a local decrease in copper associated with the synaptosomal fraction of brain [7,12]. It has been postulated that during prion disease, conversion of PrP^C into PrP^{Sc} might lead to some functional loss normally associated with this protein and may render neurons more susceptible to neurotoxicity from oxygen radicals or glutamate [13]. This has already been demonstrated in cell culture models [14]. As loss of copper binding to PrP^C is likely to result in PrP lacking the functions we have previously detected *in vitro*, we investigated whether there are changes in trace elements in the brains and other organs of mice infected with mouse prion inoculum.

METHODS

Infection of mice

C57BL/6 male and 129Sv female mice were purchased from Harlan UK (Shaw's Farm, Bicester, Oxon., U.K.) and cross-bred in-house to generate F1 wild-type progeny that were used in the present study. Mice were inoculated with 4×10^5 Rocky Mountain Laboratory (RML) prions (50% of lethal dose; a gift from Professor Adriano Aguzzi, Institute of Neuropathology, University Hospital of Zürich, Zürich, Switzerland) at 6 weeks of age via the intracerebral route in a volume of 20 μ l. Five mice per group for both the infected and uninfected controls were killed at day 0 (2 h post-inoculation), or days 30, 60, 97, 127, 150 and 160 post-inoculation. Mice taken at days 150 and 160 were all at the terminal stage of the disease. Thus in the Results and discussion section 'days post-inoculation' refers to the time at which the infected and uninfected (control) mice were killed.

Analysis of metal content

Tissue samples of liver, brain and muscle were analysed for metal content. The analysis was blinded (i.e. samples were coded, but the operator was unaware of the code), but control and scrapie-infected samples were analysed in parallel. Samples were added to pre-weighed Teflon microwave vessels, which were then reweighed to determine the weight of the samples. Liver and muscle samples were digested in 5 ml of nitric acid (Romil SpA grade) in 125-ml vessels that had been acid-leached under pressure. Samples were left overnight at 24 °C, then sealed and heated under pressure and temperature control to 120 lb/in² (where 1 lb/in² = 6.9 kPa) and at least 190 °C for 10 min. The resulting digests were clear when diluted to 25 ml with water (Elga UHQ grade), and were stored in plastic sample tubes. Brain samples were much smaller than muscle or liver samples and were digested in 7-ml microvessels using 1 ml of nitric acid under similar conditions to those used for liver and muscle, and were then diluted to 5 ml with water. All samples were

Abbreviations used: BCA, bicinchoninic acid; NBT, Nitro Blue Tetrazolium; PrP, prion protein; PrP^C, normal cellular PrP; PrP^{Sc}, abnormal disease-specific conformation of PrP; RML, Rocky Mountain Laboratory; SOD, superoxide dismutase; TMAH, tetramethylammonium hydroxide.

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subsequently stored as 20% (v/v) nitric acid digests. The samples were analysed on a PerkinElmer Plasma 40 emission ICP (Inductively Coupled Plasma) instrument without further dilution. The standards, blanks and wash solutions also contained 20% (v/v) nitric acid to avoid matrix mismatch during the analysis. All samples were analysed against a single standard solution containing 10 p.p.m. iron, 5 p.p.m. zinc, 0.5 p.p.m. copper and 0.1 p.p.m. manganese diluted from certified single element 1000 p.p.m. concentrates (Qm_x Laboratories, Thaxted, Essex, U.K.). Instrument performance was checked after every 10 samples. The measured p.p.m. values were then converted into metal concentrations in the original solid to enable comparisons to be made.

Sample preparation for protein and blood

Protein samples were adjusted to the same concentration following analysis with the bicinchoninic acid (BCA) assay (Sigma) using a standard curve of 0–100 µg of recombinant mouse PrP. Whole blood was kept alkaline to avoid clotting. The thawed blood was transferred to a tared, stoppered polystyrene test tube using a Pasteur pipette and weighed (approx. 0.50 g). Tetramethylammonium hydroxide [TMAH; 0.5 ml, as a 25% (w/v) aqueous solution; Alfa Aesar, Royston, Herts., U.K.; electronics grade] was added, and the sample was left to digest at 24 °C for at least 10 min. A 0.05 ml aliquot of solution containing 10% (w/v) sodium EDTA in the same TMAH matrix was added to aid retention of the metals in solution. An internal standard containing 1000 p.p.b. gallium, rhodium, holmium and bismuth (0.05 ml) was added and the solution was made up to 5 ml with diluent containing 0.1% (v/v) Triton X-100 surfactant (Romil SpA grade) in water (Elga UHQ grade). The 5 ml solution was thoroughly vortex-mixed prior to analysis. A ThermoElemental PQ2+ICP mass spectrometry (MS) instrument (ThermoElemental Ltd, Winsford, Cheshire, U.K.) was used for the analysis of manganese, copper and zinc. Quantitative multi-element calibrations were made using standards prepared from certified single element 1000 p.p.m. standard solutions. Zinc was calibrated at 0, 100, 200, 300, 400 and 500 p.p.b., copper was calibrated at 0, 10, 20, 30, 40 and 50 p.p.b., and manganese was calibrated at 0, 2, 4, 6, 8 and 10 p.p.b. A Gilson 222 autosampler was used for the analysis, and solutions were aspirated for 60 s before testing for 30 s in peak jumping mode with three replicates, then washing in the sample matrix for 110 s between solutions. A 10 p.p.b. standard check solution was analysed after each set of 10 samples to monitor the analysis. The internal standard elements were monitored in case the aerosol of dissolved blood partially blocked the torch injector.

Purification of PrP

PrP was purified on a bead-immobilized polyclonal anti-PrP affinity column [10]. The IgG fraction of a rabbit polyclonal antiserum raised against mouse PrP was isolated using Protein A-Sepharose (Sigma). The antiserum was raised against a peptide corresponding to amino acid residues 89–103 of the mouse PrP (Cymbus Biotechnology, Chandlers Ford, Hants., U.K.). Its specificity for the PrP was tested by ELISA (against the peptide antigen) and by comparing its specificity for the whole protein with known specific antibodies (results not shown). The IgG fraction was coupled to CNBr-activated beads (Amersham Biosciences) and used for the preparation of an affinity column with a 3 ml bed volume. Extracts were prepared from control and infected mouse brains by homogenization in PBS containing 1% (v/v) Nonidet P40 and 0.5% Tween 20 at 24 °C for 20 min

followed by vortex-mixing. Solid material was spun out at 17530 g. The volume of the extract was increased 10-fold by dilution with PBS. This extract was passed over the bead-immobilized antibody column, and the column was subsequently washed with wash solution (50 mM NaCl and 50 mM NaH₂PO₄, pH 8.0). The specifically bound material was eluted in 250-µl steps with elution buffer (50 mM glycine and 50 mM NaCl, pH 3.0), then neutralized by addition of 0.1 vol. of 100 mM Tris/HCl (pH 8.0). The initial fractions containing PrP were measured to be pH 5–6. The protein concentration was determined using the BCA assay (Sigma), and the purity of the protein was confirmed by running on a polyacrylamide gel. The effectiveness of this immunoprecipitation technique to isolate PrP, which is a standard protocol in our laboratory, has been verified on a number of occasions in the past (using silver stain) and has been reported in several papers [10,15,16]. This purification procedure did not discriminate between different forms of PrP, implying that PrP purified using this method was a mixture of PrP^c and PrP^{sc}.

SOD assay

SOD activity in brain homogenate (20 µg/ml) and from affinity-purified PrP (1 µg/ml) was determined as described previously [10,11] employing superoxide production from xanthine oxidase and detection of a coloured formazan product formed from Nitro Blue Tetrazolium (NBT) at 560 nm. The SOD activity was expressed as percentage inhibition of formazan produced, where 100% formazan product formation is the amount of NBT reduced by xanthine oxidase-formed radicals in control reactions without brain extracts or affinity-purified PrP. For manganese-SOD activity measurement, PrP-depleted brain extracts were incubated at 24 °C in 4 mM KCN for 20 min before assaying. Copper/zinc-SOD activity was calculated by subtracting manganese-SOD activity from total SOD activity in the PrP-depleted brain homogenates. All assays were performed in triplicate. The extracts used were tested for their ability to reduce NBT in the absence of xanthine oxidase. None of the extracts showed any effect on NBT conversion into formazan, as measured spectrophotometrically for 5 min. Also, NBT was reduced by xanthine oxidase to the formazan product aerobically by the addition of 50 µM xanthine to the reaction mixture but no NBT-formazan was produced in the absence of xanthine, as determined spectrophotometrically.

Proteinase K analysis

Brain extracts or affinity-purified native PrP were assessed for protease sensitivity by treatment with 250 µg/ml proteinase K (Boehringer Mannheim) at 37 °C for 1 h. The reaction was stopped by the addition of Perfabloc (Boehringer Mannheim; final concentration of 2 mM).

Western-blot analysis

Brain extracts or purified proteins were electrophoresed on a 15% (w/v) polyacrylamide gel in the presence of SDS and reducing agents. The protein was blotted on to PVDF membrane and protein was detected as previously described [10] by a specific monoclonal antibody raised against a peptide corresponding to amino acid residues 142–160 of the mouse PrP.

Statistical analysis

Statistical analysis was performed using the Student's *t* test.

Table 1 Iron and zinc in tissues from scrapie-infected mice

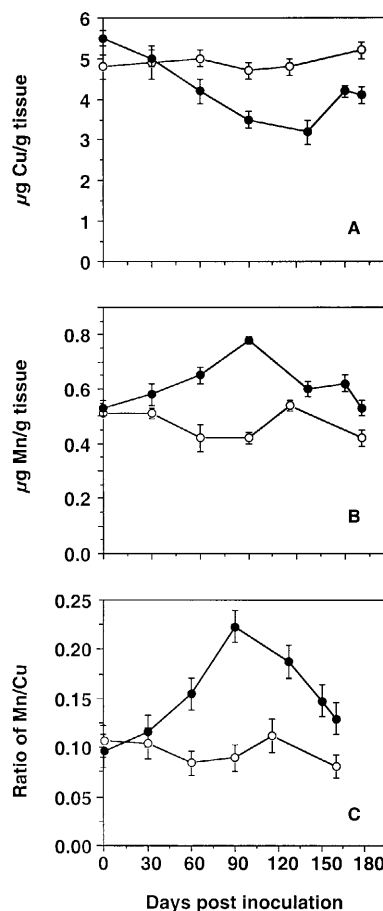
Results are presented as μg of metal/g of tissue except for blood. Iron is total iron. For blood, values are μg of zinc/g dry weight of blood. Iron was not determined for blood because of the high variability between individuals. Control values are not shown. However, there was no significant difference ($P > 0.05$) for either metal between the samples for which there was a significant difference ($P < 0.05$) between the value for the infected mice and the appropriate uninfected control. Results are presented as means \pm S.E.M. ($n = 5$).

Tissue	Metal	Days post-infection						
		0	30	60	97	127	150	160
Brain	Iron	25.0 \pm 2.0	24.4 \pm 2.2	22.0 \pm 3.0	18.1 \pm 3.2	24.0 \pm 2.0	24.2 \pm 3.2	25.6 \pm 2.3
	Zinc	18.8 \pm 2.2	20.6 \pm 2.5	18.6 \pm 2.2	16.9 \pm 2.2	18.2 \pm 2.4	18.0 \pm 2.3	18.7 \pm 3.0
Liver	Iron	150.0 \pm 10.0	150.0 \pm 15.0	143.0 \pm 20.0	140.0 \pm 10.0	125.0 \pm 12.0	114.0 \pm 35.0	162.0 \pm 25.0
	Zinc	37.0 \pm 3.0	30.0 \pm 3.0	30.0 \pm 3.0	32.0 \pm 3.0	30.0 \pm 2.0	34.0 \pm 3.0	34.0 \pm 3.0
Muscle	Iron	15.0 \pm 1.2	14.6 \pm 1.1	13.5 \pm 1.3	17.0 \pm 1.2	18.0 \pm 1.2	23.0 \pm 1.2*	22.0 \pm 1.5*
	Zinc	11.0 \pm 0.6	10.5 \pm 0.5	12.2 \pm 0.4	10.2 \pm 0.4	10.5 \pm 0.5	11.5 \pm 0.6	11.5 \pm 0.6
Blood	Iron	4484.0 \pm 340.0	3815.0 \pm 380.0	4099.0 \pm 340.0	4780.0 \pm 272.0	4845.0 \pm 224.0	5654.0 \pm 272.0*	5628.0 \pm 64.0*
	Zinc							

RESULTS AND DISCUSSION

Wild-type mice were inoculated with the RML mouse scrapie strain. Uninfected control mice were housed alongside the infected animals. Five mice per group were killed approximately every 30 days, and blood, brain, muscle and liver samples were collected and snap frozen on solid CO_2 . Samples were also collected from mice at a zero time point (2 h post-inoculation), and some were sampled at the terminal stage of the disease on days 150–160 post-inoculation. Visible clinical signs became apparent from 125 days post-inoculation and continued until day 160, when all remaining mice were killed at the terminal stage of the disease. Different sample time points appear between prion-infected and control mice in the latter part of the experiment due to the fact that some of the infected mice reached the terminal stage of the disease prior to the intended sample time point and had to be killed.

MS was used to assess the levels of metals in the different tissues. In particular, copper, manganese, zinc and iron were examined. Metal concentrations in tissues were assessed in terms of changes associated with disease progression and onset of

**Figure 1** Metal changes in the brain

ICP-MS was used to measure the metal content of the brains of mice infected with RML and control mice of similar ages. The levels of copper (A) and manganese (B) in infected mice were determined and plotted according to the time after inoculation (\bullet). The control mice of similar age, killed at similar time points, are plotted on the same graphs (\circ). The relative levels of manganese compared with copper in the brains of the mice were compared by dividing the values for manganese by those of copper. These values are plotted in (C). Results are presented as means \pm S.E.M. ($n = 5$).

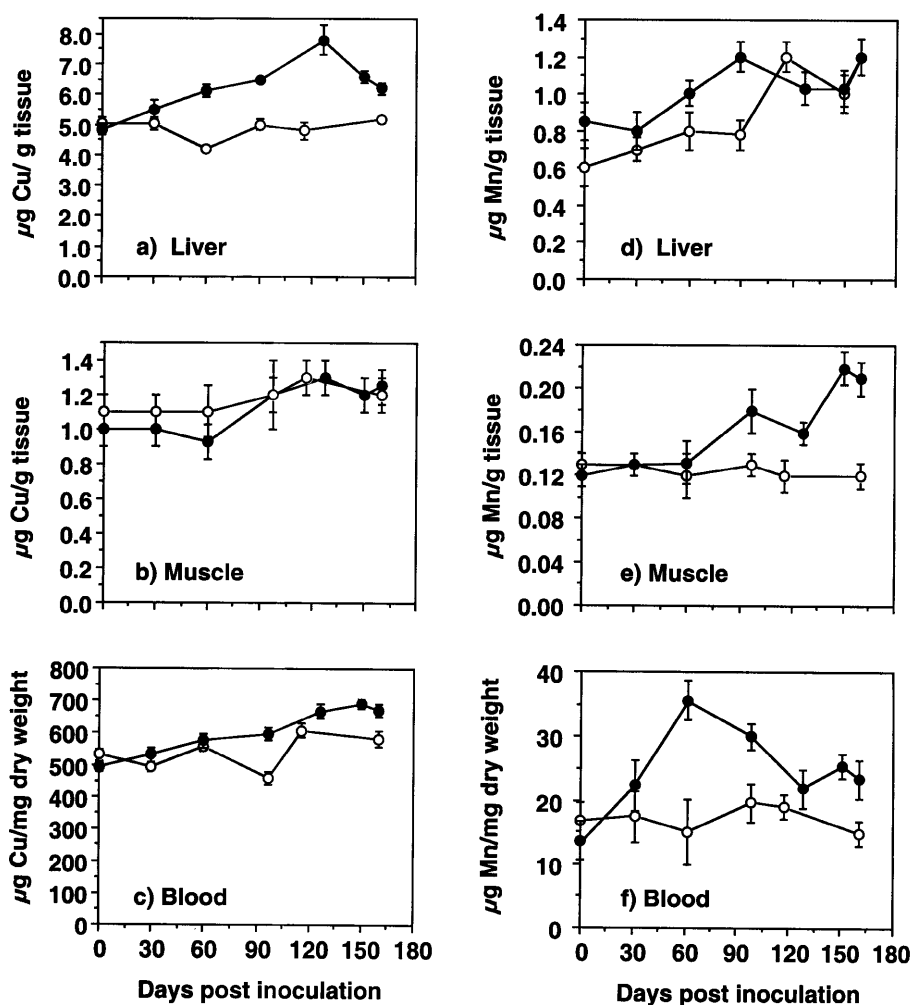


Figure 2 Changes in manganese and copper in other tissues

ICP-MS was used to measure the metal content of the liver, muscle and blood of RML-infected and uninfected control mice. Copper was measured in liver (a), muscle (b) and blood (c), and manganese was measured in liver (d), muscle (e) and blood (f). Values for infected (●) and uninfected (○) control mice were plotted according to the number of days post-inoculation. Results are presented as means \pm S.E.M. ($n = 5$).

clinical signs. There were no changes in either zinc or iron for liver (Table 1) compared with samples from control mice of similar age ($P > 0.05$). Blood showed a minor increase in zinc, and muscle showed a small increase in iron in terminal prion-diseased animals only.

Changes were observed for copper in samples of brain, liver and blood. In particular there was a reduction in brain copper (Figure 1) that first showed significance ($P < 0.05$) at 97 days post-inoculation, i.e. before the onset of clinical signs, and remaining so until the terminal stages of the disease. In liver, copper was found to be significantly elevated ($P < 0.05$) from a time point of 60 days post-inoculation (Figure 2). The copper levels in blood were also significantly different at days 97 and 127 ($P < 0.05$). This suggested that there was a displacement of copper from the brain or other tissues. There was no significant change in copper in muscle at any time point ($P > 0.05$).

Changes in manganese in tissues of scrapie-infected mice were more widespread. Elevated manganese levels were first observed in blood (Figure 2; significant at days 60, 97, 160; $P < 0.05$) and later in both brain (Figure 1; significant from day 60 onwards; $P < 0.05$) and muscle (Figure 2; significant from day 90 on-

wards; $P < 0.05$). It was particularly interesting to note a decrease in copper and an increase in manganese in the brain. The relative change in the ratio of manganese to copper reached its maximum at 90 days post-inoculation at the time of onset of clinical signs (Figure 1). Manganese poisoning or manganism are the only diseases associated with changes in tissue manganese levels [17,18]. Our findings in the present study are the first report of a neurodegenerative disease in which there is a systemic change in manganese. As such, these changes may represent a characteristic that will allow selective diagnosis of prion disease. Possibly, if such changes occurred in humans they may represent a preclinical warning for the onset of prion disease.

It has been suggested that PrP^c is an antioxidant protein. Studies of mice infected with scrapie have already provided evidence for an increase in markers for oxidative stress [19]. We looked to see if there were changes in antioxidant proteins associated with disease progression in mouse scrapie. Protein extracts were prepared from scrapie-infected and control mice at various time points post-inoculation. The extracts were used for immunopurification of PrP. Western-blot analysis confirmed that the procedure led to the isolation of PrP. The protein

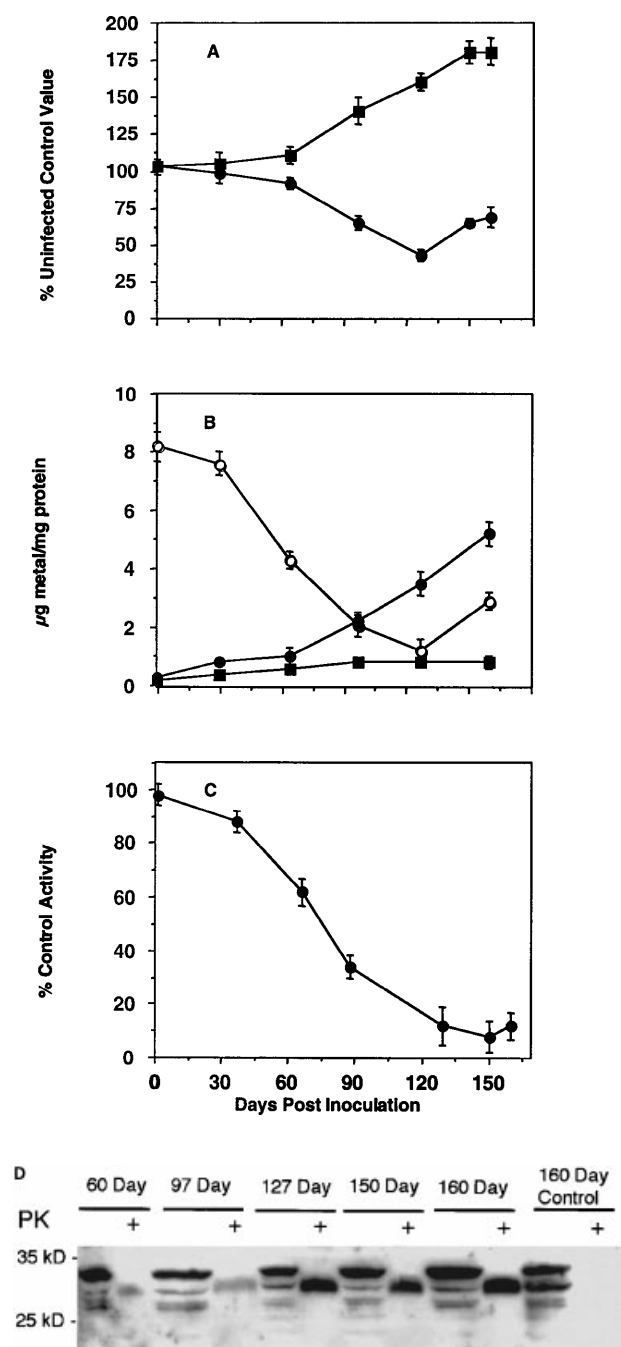


Figure 3 Changes in antioxidant proteins

(A) The level of activity of copper/zinc-SOD (●) and manganese-SOD (■) in protein extracts from the brains of RML-infected mice at different times after inoculation compared with the activity in age-matched, uninfected controls as a percentage. Results are presented as means \pm S.E.M. ($n = 5$). (B) Metal content of affinity-purified PrP protein from the brains of RML-infected mice as determined by MS. Copper (○), manganese (●) and zinc (■). Results are presented as means \pm S.E.M. ($n = 3-5$). (C) SOD activity of PrP affinity-purified from RML-infected mouse brain compared with the activity of age-matched, uninfected controls as a percentage. The activity of PrP was determined using the NBT/xanthine oxidase spectrophotometric assay. Results are presented as means \pm S.E.M. ($n = 5$). (D) Western-blot analysis of affinity-purified PrP from the brains of RML-infected mice and control mice before and after proteinase K (PK) digestion. For proteinase K digestion, samples were incubated with 250 μ g/ml proteinase K for 1 h. After this time Perfabloc (0.5%) was added to the samples. PrP purified from mice 0 or 30 days after inoculation showed no proteinase K-resistant band. kDa, kDa.

content of the preparation was accurately determined by a spectrophotometric assay. The level of copper/zinc-SOD activity (SOD-1) and manganese-SOD activity (SOD activities unrelated to PrP) in scrapie-infected mice was assessed using PrP-depleted extracts, in comparison with control mice (Figure 3A). Standard assay techniques were used to detect SOD activity in the extracts. There was a decrease in copper/zinc-SOD in extracts from scrapie-infected mice compared with control mice. This decrease was significant ($P < 0.05$) from 97 days post-inoculation, and the levels remained significantly decreased up until terminal stages of the disease. In comparison, manganese-SOD showed an increase in activity in extracts from scrapie-infected mice. The increase in the level of activity of manganese-SOD correlates with the suggestion that the brains of scrapie-infected mice are subject to increased oxidative stress [19]. Copper/zinc-SOD is known to be dependent on the availability of copper in the brain. It is possible that the decreased activity of this enzyme reflects this fact. Recently, Wong et al. [15] have shown that the protein expression level of both copper/zinc-SOD and manganese-SOD was not altered in total brain homogenate from terminally prion-infected mice both before and after immunopurifying PrP, and that total SOD activity was significantly reduced in prion-infected brains compared with controls. In addition, and similar to that shown in the present study, after immunoprecipitating PrP, the level of copper/zinc-SOD activity was reduced compared with controls, whereas manganese-SOD activity in prion-infected brains was increased [15]. Collectively, these data show that whereas protein expression levels of both copper/zinc-SOD and manganese-SOD are not altered by prion disease the activity of these enzymes is affected.

Purified recombinant and native PrP^c have been shown to possess an activity similar to that of SOD which is dependent on bound copper [10,11]. The metal content (Figure 3B) and the level of PrP-dependent SOD activity in purified protein was assessed (Figure 3C). During the course of infection, scrapie-inoculated mice displayed a rapid loss in the amount of copper bound to the protein (significant from day 60 onwards; $P < 0.05$) and a gradual increase in the amount of manganese associated with PrP purified from infected mouse brain (significant from day 97 onwards; $P < 0.05$). The same purified PrP was subjected to a Nitro Blue Tetrazolium/xanthine oxidase-based assay of SOD activity. In comparison with PrP from age-matched, uninfected controls there was a marked decrease in SOD-like activity associated with PrP from the brains of infected mice. This was significant from 30 days post-inoculation onwards ($P < 0.05$). The decrease in activity matched the decrease in copper content of the protein. Analysis of the proteinase K resistance of PrP purified from the brains of the infected mice showed that the procedure led to the acquisition of a mixture of proteinase-resistant and -sensitive material and that a significant amount of the protein was proteinase-resistant from 60 days post-inoculation. This result implies that there is a reduction in copper and antioxidant activity of the total PrP content that was affinity-purified from infected mouse brains.

Previous work has shown that manganese binding to PrP^c leads to its conversion into an abnormal form of PrP rich in β -sheet and with resistance to proteinase K digestion [20]. It was suggested that an imbalance in brain trace elements, such as an excess of manganese and a loss of copper, might result in conditions that lead to the formation of PrP^{sc}. The results shown in the present study suggest that RML-induced prion disease might cause such a change in the brain. Therefore it is possible that metal imbalance could cause conversion of PrP^c into PrP^{sc}. Formation of PrP^{sc} was associated with a loss of copper binding and a reduction in the antioxidant activity of the protein. These

changes precede clinical signs and could be involved in the onset of the pathology associated with this disease. It has been suggested that loss of function of PrP^c does not contribute to prion disease because mice devoid of PrP^c expression [21] lack a clinical phenotype. However, it should be noted that prion disease does not occur in the absence of PrP^c [6]. Therefore it is possible that the presence of non-functional PrP can inhibit compensatory mechanisms that replace PrP function in mice devoid of PrP^c expression. Indeed, there is evidence that the presence of PrP^c that cannot be converted into PrP^{Sc} prevents the neurodegenerative effects of non-host specific PrP^{Sc} [22].

It has been suggested that changes in environmental trace elements in the diet could initiate or increase the incidence of sporadic prion disease [20]. Although this theory is unverified it is clear from these results that prion disease may cause an early change in the level of copper and manganese in the body and especially the brain of infected animals. The changes in muscle and blood require further investigation, but the implication is that these values might represent specific alterations with immediate diagnostic potential. Our data suggest that the balance of manganese and copper in RML-infected animals is central to the disease process, precedes clinical signs and might cause or be symptomatic of both the onset of symptoms and the neurodegeneration characteristic of prion disease.

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