Detection of proteinase K-resistant prion protein and infectivity in mouse spleen by 2 weeks after scrapie agent inoculation

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The sequential accumulation of the protease-resistant form of the endogenous prion protein (PrP-res) was compared to levels of scrapie infectivity in the spleen and brain of scrapie-infected mice at various times after inoculation. In mouse spleen PrP-res was detected 1 week after inoculation, and increased 65-fold between 1 and 3 weeks post-inoculation and an additional 15-fold during the next 17 weeks. Infectivity in spleen reached a maximum plateau level by 3 weeks. In contrast, in mouse brain PrP-res was not detected until 8 weeks after inoculation and then increased 200-fold during the next 12 weeks. During this same time, infectivity increased approximately 10000-fold. Therefore, in both spleen and brain of scrapie-infected mice accumulation of PrP-res and infectivity appear to be associated. However, it was not possible to show quantitative correlations between PrP-res detection and infectivity, perhaps owing to the inaccuracy of the infectivity assay.

Spongiform encephalopathies of man and animals are caused by unique transmissible agents that are unusually resistant to chemical inactivation and treatments that destroy or modify nucleic acid (Alper et al., 1967; Bellinger-Kawahara et al., 1987; Dees et al., 1985; Latarjet, 1979). In preparations enriched for infectivity, a predominant proteinase K-resistant form (PrP-res) of the endogenous prion protein (PrP) has been identified (Bolton et al., 1982; Diringer et al., 1983; Hope et al., 1986). Several investigators have sought to determine the relationship between PrP-res and the causative agent. McKinley et al. (1983) and Jendroska et al. (1991) have shown a reasonably close association between the detection of PrP-res and infectivity in the brains of scrapie-infected hamsters, supporting the notion that the protein itself could be the scrapie agent. However, their data conflict with those reported by Czub et al. (1986) which show that infectivity reaches maximum levels well before significant PrP-res is detected, a finding which led to the conclusion that PrP-res is not the scrapie agent. Rubenstein et al. (1991) detected PrP-res in the spleens of scrapie-infected mice 11 to 12 weeks post-inoculation (p.i.), which again is well after infectivity levels in spleen plateau. However, Doi et al. (1988) were able to detect PrP-res in mouse spleen by 5 weeks p.i., making the association with infectivity more likely, but they did not determine infectivity. Considered together, these data show wide variation with regard to the time p.i. at which PrP-res is detected in animal tissues. Consequently, interpretations of how PrP-res and the scrapie agent might be associated vary.

Since we have developed a sensitive technique for detecting PrP-res in mouse spleen, we wondered whether it might be possible to detect PrP-res much earlier p.i. than has been possible previously. If so, this detection might more accurately define possible associations between PrP-res and infectivity. We were particularly interested in evaluating PrP-res in mouse spleen because scrapie agent titres increase rapidly in the spleens of mice within days of inoculation and, in contrast to brain, do so in the absence of histopathological changes.

To determine how closely PrP-res and infectivity are associated in mouse spleen we analysed the spleens from mice killed at various times p.i. Rocky Mountain Laboratories (RML) random bred Swiss mice were inoculated intracerebrally with 50 μl of a 1% suspension of brain from clinically sick scrapie-infected mice (Chandler isolate). Groups of mice were killed at 1 day and at 1, 2, 3, 8 and 20 weeks p.i. Their spleens and brains were harvested aseptically and frozen until they could be analysed. PrP-res preparations were made from pooled spleens using several modifications of a technique described previously (Doi et al., 1988). Briefly, 10% suspensions of spleen in 0.01 M-Tris-HCl, 0.005 M-MgCl₂ were made by forcing the spleens through a fine mesh stainless steel screen. These suspensions, excluding the fibrous material left on the screen, were then treated with DNase (400 μg/g original tissue) for 1 h at 37 °C. An
equal volume of 20% sarkosyl in 0.01 M-Tris–HCl pH 7.6 was then added and the suspension was maintained at room temperature for 30 min. The suspension was then centrifuged at 10,000 g for 30 min. Supernatants were recovered and centrifuged at 21,500 g for 2 h. The pellets were resuspended in 1.0 ml of sterile glass-distilled H2O by sonication. Proteinase K (10 μg/g of original tissue) was added and the suspension kept at 37 °C for 30 min. PMSF was added to a final concentration of 0.005 M. This suspension was kept on ice for 30 min then centrifuged at 21,500 g for 1 h. The pellet was suspended in sample buffer (Laemmli, 1970) containing 5% SDS at a concentration of 20 mg/μl (based on original tissue weight) and frozen.

PrP-res was prepared from brain using the same procedure except that the initial 10% homogenates were made using a Dounce homogenizer. Proteins were separated on polyacrylamide gels and the PrP-res bands detected as described (Race et al., 1992).

For ease of understanding, we express PrP-res as units (U)/100 mg of tissue. This value was determined by dividing 100 by the minimum mass (mg) of tissue needed to detect PrP-res by immunoblotting. On this basis, 0.2 U of PrP-res/100 mg was detected in spleen 1 week after inoculation. However, detection of 0.2 U at 1 week required that 500 mg equivalents of spleen or approximately five mouse spleen equivalents be analysed by immunoblotting (Fig. 1, lane 2). By 3 weeks after inoculation, 13 U/100 mg was detected, a 65-fold increase over the amount detected at 1 week post-inoculation. Another 15-fold increase in the amount of PrP-res occurred between 3 and 20 weeks p.i. (Table 1 and Fig. 1). No PrP-res was detected 1 day p.i. when 2000 mg equivalents of spleen (approximately 20 mouse spleen equivalents) were analysed, suggesting that the

Table 1. Association between PrP-res accumulation and infectivity in mouse spleen and brain

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time p.i. (weeks)</th>
<th>PrP-res U*/100 mg tissue</th>
<th>LD50/100 mg tissue†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>1</td>
<td>0.2</td>
<td>6.0 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.8</td>
<td>1.1 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.0</td>
<td>6.0 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>50.0</td>
<td>1.9 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>200.0</td>
<td>1.2 × 10⁷</td>
</tr>
<tr>
<td>Brain</td>
<td>1</td>
<td>ND‡</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;0.2</td>
<td>1.2 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>13.0</td>
<td>1.8 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2500.0</td>
<td>1.9 × 10⁹</td>
</tr>
</tbody>
</table>

* The minimum amount of spleen or brain equivalents needed to detect PrP-res at the various times p.i. was determined by immunoblotting (Fig. 1 and 3) and was divided into 100 to give the number of PrP-res U in a standard weight of tissue (100 mg).
† The amount of infectivity in the original 10% homogenates from which PrP-res was recovered was determined as described (Eklund et al., 1967; Race et al., 1992).
‡ ND, Not done.
PrP-res detected in spleen 1 week p.i. was not derived from the inoculum (data not shown). However, when an amount of brain-derived inoculum equivalent to the amount used to inoculate five mice was purified as the spleens were, and then immunoblotted as before, bands representing PrP-res were detected (Fig. 2, lane 5). Thus, the PrP-res detected at 1 week p.i. could, at least in part, have been derived from inoculum if all of the intracerebrally inoculated PrP-res were sequestered in the spleen by 1 week p.i. (compare Fig. 1, lane 2 with Fig. 2, lane 5). By 2 weeks p.i. the intensity of the bands from an equivalent amount of spleen exceeded that of the inoculum (compare Fig. 2, lane 5 with Fig. 1, lane 5). Therefore, by 2 weeks p.i. the PrP-res bands which were observed from the inoculum (data not shown). However, when an amount of brain inoculum equivalent to the spleen sample from infected mice were analysed (Fig. 3). Nor was PrP-res detected 1 day p.i., when 2000 mg equivalents of spleen were analysed (data not shown). However, at 8 weeks p.i., PrP-res was detected with as little as 7.8 mg equivalents of brain. Between 8 and 20 weeks p.i. the amount of PrP-res increased approximately 200-fold. However, during this interval the amount of infectivity in brain increased 10000-fold (Fig. 3, Table 1). Thus, there was a 50-fold difference between the increase in PrP-res and the increase in infectivity between 8 and 20 weeks p.i.

From the data presented here it seemed reasonable to conclude that PrP-res and infectivity are associated, in that PrP-res was never detected in the absence of infectivity. However, directly related quantitative increases were not apparent. We wondered whether the discrepancy between increases in PrP-res and infectivity which was observed after 3 weeks in spleen and between 8 and 20 weeks in brain could be due to the margins of error encountered in either the infectivity titrations or PrP-res analyses. Therefore, we investigated the potential infectivity titration error by determining the infectivity titres of eight different but equivalent spleen homogenates and three brain homogenates. The range in titre was 1.7 × 10⁴ to 6.0 × 10⁵ LD₅₀/mg for spleen and 1.8 × 10⁹ to 1.9 × 10⁷ LD₅₀ for brain (data not shown). Thus there appeared to be a 10- to 15-fold range of difference in infectivity titrations on comparable samples.

To determine the potential error which one might expect from the immunoblot analysis, we analysed PrP-res preparations derived from aliquots of a single
pool of mouse spleens taken from mice killed 3 weeks after inoculation of scrapie agent. PrP-res was prepared on different days and immunoblotted on different days but using the same techniques. All values were within a twofold range of each other. Thus, the immunoblot analysis for PrP-res had a much lower potential for error than the infectivity analyses. Nevertheless, the combined potential errors of the infectivity and PrP-res analyses could possibly account for the 15-fold increase in PrP-res between 3 and 20 weeks p.i. in spleen. However, the 50-fold difference between PrP-res and infectivity increases in brain which occurred between 8 and 20 weeks p.i. appeared to exceed the range of error observed.

Reports prior to this have shown that whenever PrP-res is detected, infectivity is also present provided agent and PrP-res represent the same species (Jendroska et al., 1991; Rubenstein et al., 1991; McKinley et al., 1983; Czub et al., 1986). However, in all instances detection of infectivity preceded the demonstration of PrP-res accumulation. In the one report comparing PrP-res and infectivity in mouse spleen, infectivity was detected approximately 9 weeks before PrP-res, leading the authors to conclude that PrP-res and infectivity increase independently (Rubenstein et al., 1991). We detected de novo synthesis of PrP-res by 2 weeks p.i., which is by far the earliest that PrP-res has been detected in mice, an observation which makes the association between PrP-res and infectivity stronger than before. However, after a few weeks the association is less precise and our data comparing PrP-res and infectivity after 3 weeks failed to show a quantitative correlation between PrP-res and infectivity.

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References


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