Molecular analysis of iatrogenic scrapie in Italy

Gianluigi Zanusso,1 Cristina Casalone,2 Pierluigi Acutis,2 Elena Bozzetta,2 Alessia Farinazzo,1 Matteo Gelati,1 Michele Fiorini,1 Gianluigi Forloni,3 Man Sun Sy,4 Salvatore Monaco1 and Maria Caramelli2

1Department of Neurological and Visual Sciences, University of Verona, Piazzale L. A. Scuro, 10, 37134 Verona, Italy
2CEA Istituto Zooprofilattico del Piemonte, Liguria e Valle d’Aosta, Via Bologna 148, 10154 Torino, Italy
3Laboratorio di Biologia delle Malattie Neurodegenerative, Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milano, Italy
4Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106-1712, USA

An accidental intra- and interspecies transmission of scrapie occurred in Italy in 1997 and 1998 following exposure to a vaccine against Mycoplasma agalactiae. PrPSc in affected sheep and goats, collected from a single flock exposed to vaccination 2 years earlier, was molecularly typed. In five animals with iatrogenic scrapie, a PrPSc type with a 20 kDa core fragment was found in all areas of the brain investigated. In three sheep and one goat, this isoform co-occurred with a fully glycosylated isoform that had a protease-resistant backbone of 17 kDa, whereas in two sheep and four goats, the two PrPSc types were detected in different regions of the brain. In sheep with natural field scrapie, a PrPSc type with physico-chemical properties indistinguishable from the 20 kDa isoform was found. The present results suggest the co-presence of two prion strains in mammary gland and brain homogenates used for vaccination.

INTRODUCTION

Transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative diseases, which include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans (Prusiner, 1997). These disorders are characterized by a post-translational conversion and brain accumulation of an insoluble, protease-resistant isoform (PrPSc) of the host-encoded prion protein (PrPC). Several TSE strains have been isolated in both animal and human disorders. Upon experimental propagation, prion strains are distinguished according to differences in incubation time, regional distribution of neuropathological lesions and distinct conformations of the pathological protein (Bruce et al., 1991; Bessen & Marsh, 1994). The emergence of cattle BSE and the evidence that its agent has spread in a variety of mammalian species, including humans, has raised concerns about the presence of BSE in other animals in field conditions, such as sheep and goats (Bruce et al., 1997; Hill et al., 1997). Sheep BSE is reported to be clinically indistinguishable from scrapie (Foster et al., 1993). Since sheep-adapted BSE strains are potentially infectious for animals and humans, a strict surveillance for BSE in sheep and goat scrapie is currently required. To date, at least 14 distinct strains of scrapie have been identified by serial propagation in mice (Bruce et al., 1991; Bruce, 1993), with recent evidence indicating that the spectrum of natural scrapie strains in sheep may have changed over the last two decades (Bruce et al., 2002). Conventional strain typing is, however, not feasible on large-scale cases and screening for strain differentiation must rely on physico-chemical properties of PrPSc. Biochemical evidence of strain variation in historical, contemporary and experimental scrapie has been provided (Hill et al., 1998; Hope et al., 1999) and a molecular classification of PrPSc types has been proposed, based mainly on the molecular mass of proteinase K (PK)-resistant core fragment and glycoform profiles (Hope et al., 1999).

Over the last 15 years, clinical, genetic and pathological studies of field sheep and goat scrapie, as well as of iatrogenic scrapie, have been reported in Italy (Mechelli & Mantovani, 1988; Capucchio et al., 1998, 2001; Vaccari et al., 2001). However, strain typing and molecular PrPSc characterization studies in Italian scrapie are still lacking.

The aim of this study was to perform a molecular characterization of PrPSc of sheep and goats collected from a single Italian flock with iatrogenic scrapie and to compare physico-chemical properties of PrPSc types with Italian field scrapie.
METHODOLOGY

Animals. In Italy, a sudden increase in outbreaks of confirmed cases of scrapie was observed between August 1996 and October 1997 in sheep and goats vaccinated previously against Mycoplasma agalactiae (Agrimi et al., 1999). In January 1999, a new outbreak was reported in a mixed flock of Comisana sheep and half-bred goats exposed to the same vaccine, with all available evidence that the epidemic represented a further iatrogenic form of scrapie (Caramelli et al., 2001). A total of 15 animals from a single flock exposed to the vaccine, including nine sheep and six goats, were available for the present study. All sheep PrP genotypes were A136RR154QQ171 and all goats were H142HH143SP240 (Goldmann et al., 1996). In addition, three unvaccinated sheep of the same breed and genotype with natural scrapie were analysed.

Sample preparation. Different areas of the brain, including the brainstem, thalamus and cortex, were obtained. Each sample was dissolved in 9 vols of lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40 and 10 mM EDTA in TBS) at pH 4, 7 and 8, respectively, and clarified by centrifuging at 10,000 g for 10 min. The supernatant was stored at −80°C until use; the pellet was discarded.

PK and N-glycosidase F treatments. Protease resistance was assayed by incubating aliquots of brain homogenate with 50 µg PK ml−1 (Boehringer Mannheim) at 37°C for 1 h. Digestion was blocked by the addition of PMSF to 3 mM. For N-deglycosylation, samples were treated with N-glycosidase F (PNGase F), according to the manufacturer’s instruction (Boehringer Mannheim).

Immunoblot analysis. For SDS-PAGE, sample aliquots were dissolved in sample buffer (3% SDS, 3% β-mercaptoethanol, 2 mM EDTA, 10% glycerol and 62.5 mM Tris, pH 6.8) and boiled for 5 min. An equivalent of 0.5 mg of wet tissue was loaded on 12% SDS-polyacrylamide gels and, after separation, proteins were transferred onto PVDF membranes (Immobilon-P, Millipore) for 2 h at 60 V. Membranes were blocked with 1% non-fat dry milk in TBST (TBS with 0.1% Tween 20) and incubated overnight at 4°C with anti-PrP antibody 8C6 (1:1000), which recognizes the amino acid sequence 145–167 (Zanusso et al., 1998). After washing, the membranes were incubated with a peroxidase-conjugated anti-mouse IgG, developed using an enhanced chemiluminescence system (ECL, Amersham) and visualized using Biomax MR films (Eastman Kodak). Films were scanned using a densitometer (GS700, Bio-Rad) and data were analysed using Excel (Microsoft).

RESULTS

PrPSc typing in sheep with natural and iatrogenic scrapie

In all the sheep with field scrapie and in four sheep from the infected flock (group 1), immunobLOTS of brain homogenates obtained from the brainstem, thalamus and cerebral cortex showed three PK-resistant PrPSc bands, migrating at ~32, 25 and 20 kDa, corresponding to the di-, mono- and unglycosylated forms of PrP (Fig. 1a, odd lanes). No major differences in PrPSc glycosylation were observed among proteins from different areas of the brain, in keeping with the notion that TSE strain controls the glycosylation of the pathological protein (glycoform percentages are reported in Table 1). After enzymatic deglycosylation, the three PrPSc bands migrated to the 20 kDa zone (Fig. 1a, even lanes). In three sheep with iatrogenic scrapie (group 2), in addition to PK-resistant PrPSc species with molecular masses indistinguishable from those of group 1, an unglycosylated fragment, migrating at ~17 kDa, was detected in all areas of the brain investigated (Fig. 1b, odd lanes). After deglycosylation, all PrPSc forms migrated at 20 and 17 kDa, respectively, the 20 kDa PrPSc fragment being about fivefold the 17 kDa PK-resistant polypeptide. Quantification by densitometric analysis showed that the relative percentages of the di-, mono- and unglycosylated PrPSc...
forms were similar to those obtained in animals from group 1 (Table 1).

Finally, in two sheep from the infected flock (group 3), the isoforms with the 20 and 17 kDa unglycosylated PrP<sup>Sc</sup> fragments were detected in different areas of the brain. As shown in Fig. 1(c), PK digestion of brainstem homogenates generated three bands migrating at 32, 25 and 20 kDa, with glycoform ratios very similar to those detected in the former groups. Conversely, immunoblots of cerebral cortex homogenates showed the presence of PK-resistant PrP<sup>Sc</sup> forms that migrated to about ~3 kDa less than the above isoforms, all shifting to the ~17 kDa zone following PNGase treatment (Fig. 1c, lanes 3 and 4). Interestingly, in addition to differences in electrophoretic mobility, PrP<sup>Sc</sup> products obtained from cerebral cortex extracts showed a distinct glycosylation profile, since they were mainly detected as heavily glycosylated forms (Table 1 and Fig. 3).

Since sheep from all three groups were of the same breed and genotype, a role for codon 136/154/171 allotype in determining different molecular signatures between the two PrP<sup>Sc</sup> types can be confidently ruled out. In addition, it appears unlikely that both the survival times after vaccination (~24 months in all animals) and the age of the sheep at death could explain the variation of glycoform profiles and electrophoretic mobilities detected in the present study.

Although a correlation between distinct immunoblot patterns and neuropathological changes was expected in all three groups, the very low spongiform change score detected in the frontal and parietal cortices of all sheep (Caramelli et al., 2001) did not allow such an analysis. Ongoing transmission studies will elucidate the biological relevance of the present biochemical findings.

### PrP<sup>Sc</sup> typing in goats with iatrogenic scrapie

Results obtained in goats were comparable to those observed in sheep. In one goat, the PrP<sup>Sc</sup> isoform with the 20 kDa unglycosylated fragment was detected in all areas of the brain (Fig. 2a), the relative percentage of glycoforms overlapping that seen in group 1 sheep (Fig. 3). In one goat, the 20 and 17 kDa PrP<sup>Sc</sup> co-occurred in all areas of the brain (Fig. 2b), whereas in four goats, similar to group 3 sheep, the two PrP<sup>Sc</sup> types were detected in the brainstem and in the cerebral cortex, respectively (Fig. 2c). In summary, both sheep and goats from the vaccinated flock shared two identical PK-resistant PrP<sup>Sc</sup> types, which have only slightly different patterns of glycosylation (Fig. 3).

### Effect of pH on the conformation of sheep and goats PrP<sup>Sc</sup> types

To gain insight into the physico-chemical properties of PrP<sup>Sc</sup>, we also investigated the conformational stability of

## Table 1. Molecular grouping of sheep and goats

<table>
<thead>
<tr>
<th>Animal</th>
<th>Group</th>
<th>No. affected</th>
<th>Age (years)</th>
<th>Genotype</th>
<th>PrP&lt;sup&gt;Sc&lt;/sup&gt; core fragment size (kDa)</th>
<th>PrP&lt;sup&gt;Sc&lt;/sup&gt; glycoform (mean %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>1</td>
<td>4</td>
<td>5·5 (3–7)</td>
<td>AA&lt;sub&gt;136&lt;/sub&gt; RR&lt;sub&gt;154&lt;/sub&gt; QQ&lt;sub&gt;171&lt;/sub&gt;</td>
<td>20</td>
<td>H: 56-50 ± 2·50 L: 29-82 ± 1·89 U: 13-68 ± 1·61</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>4 (3–5)</td>
<td>AA&lt;sub&gt;136&lt;/sub&gt; RR&lt;sub&gt;154&lt;/sub&gt; QQ&lt;sub&gt;171&lt;/sub&gt;</td>
<td>20/17</td>
<td>H: 51-90 ± 2·98 L: 34-30 ± 2·55 U: 13-70 ± 1·20</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>4 (3–5)</td>
<td>AA&lt;sub&gt;136&lt;/sub&gt; RR&lt;sub&gt;154&lt;/sub&gt; QQ&lt;sub&gt;171&lt;/sub&gt;</td>
<td>20 (Brainstem)</td>
<td>H: 54-50 ± 2·87 L: 32-74 ± 2·26 U: 12-76 ± 2·66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17 (Cerebral cortex)</td>
<td>H: 80-40 ± 3·41 L: 16-65 ± 3·42 U: 2·95 ± 0·58</td>
</tr>
<tr>
<td>Goat</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>II&lt;sub&gt;142&lt;/sub&gt; HH&lt;sub&gt;143&lt;/sub&gt; SP&lt;sub&gt;240&lt;/sub&gt;</td>
<td>20</td>
<td>H: 61-87 L: 27-82 U: 10-31</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>II&lt;sub&gt;142&lt;/sub&gt; HH&lt;sub&gt;143&lt;/sub&gt; SP&lt;sub&gt;240&lt;/sub&gt;</td>
<td>20/17</td>
<td>H: 63-21 L: 26-02 U: 10-77</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>NR</td>
<td>II&lt;sub&gt;142&lt;/sub&gt; HH&lt;sub&gt;143&lt;/sub&gt; SP&lt;sub&gt;240&lt;/sub&gt;</td>
<td>20 (Brainstem)</td>
<td>H: 60-67 ± 2·03 L: 28-43 ± 2·21 U: 10-90 ± 1·99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17 (Cerebral cortex)</td>
<td>H: 75-27 ± 1·44 L: 16-60 ± 1·76 U: 8·13 ± 1·40</td>
</tr>
</tbody>
</table>
the PrP\textsuperscript{Sc} associated with field and iatrogenic scrapie under acidic and basic conditions. For this purpose, brain samples were homogenized in lysis buffer at pH 4·0 and 8·0 and digested with PK. After this treatment, the cleavage products of the scrapie-associated PrP\textsuperscript{Sc} glycoforms with the 20 kDa unglycosylated fragment had a molecular mass of \(~1\) kDa greater at pH 4·0 (Fig. 4a, odd lanes; Fig. 4b, lanes 1 and 5) than glycoforms obtained at pH 8·0 (Fig. 4a, even lanes; Fig. 4b, lanes 2 and 6). The changes in migration affected all glycoforms, as assessed by examination of digestion

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**Fig. 2.** Immunoblot characterization of PrP\textsuperscript{Sc} in goats with iatrogenic scrapie. Western immunoblot with 8C6 antibody of homogenates from the brainstem and cerebral cortex before (odd lanes) and after (even lanes) PNGase treatment in goats from group 1 (a), group 2 (b) and group 3 (c). As in sheep, three patterns are detected, according to the size of the core fragment and the co-occurrence of the 20 and 17 kDa PrP\textsuperscript{Sc} types. Each lane was loaded as described in Fig. 1.

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**Fig. 3.** Scattergram showing the PrP\textsuperscript{Sc} glycoform profiles in investigated sheep and goats. The high molecular mass PrP\textsuperscript{Sc} glycoform is plotted against the low molecular mass PrP\textsuperscript{Sc} glycoform.

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**Fig. 4.** Western immunoblots with 8C6 antibody of PrP\textsuperscript{Sc} following PK treatment in the region of pH 4–8 of brain homogenates representative of sheep and goats from group 1 (a) and group 3 (b). pH values of lysis buffer are indicated. The molecular mass of the 20 kDa PrP\textsuperscript{Sc} isoform is sensitive to changes in pH, whereas the 17 kDa PrP\textsuperscript{Sc} is not modified under acidic and basic conditions.
products after removal of sugar chains. On the contrary, the 17 kDa PrPSc type was unaffected by pH variations (Fig. 4b, lanes 3, 4, 7 and 8).

DISCUSSION

This study shows that brain extracts from a group of Italian sheep and goats, collected within a single scrapie-infected flock, and from animals with natural field scrapie share a PrPSc type with a PK-resistant unglycosylated fragment of 20 kDa. Also, in some sheep and goats, an additional PrPSc type with a 17 kDa core fragment was detected. Besides differences in the size of the core fragment, indicative of distinct conformations, the two PrPSc types showed different physico-chemical properties under acidic and basic conditions. As shown previously in human prion diseases (Zanusso et al., 2001), changes in PrPSc conformation under acidic and basic conditions are related to the presence of the N-terminal octarepeats and to the extent of histidine protonation. Therefore, the present results suggest that the PrPSc with the 20 kDa core fragment is present as full-length protein in its native form, whereas the 17 kDa type lacks the octarepeat domain.

Current molecular diagnosis of human and animal prion diseases relies strongly on Western blot detection of PrPSc, whereas the characterization of the prion phenotype is based mainly on the size of the core fragment and the ratio of the three PrPSc glycoforms.

Distinct PK cleavage sites of the PrPSc in different prion strains were demonstrated first in two agents responsible for transmissible mink encephalopathy (TME), named hyper (HY), with a 21 kDa PrPSc unglycosylated fragment, and drowsy (DY), with a 19 kDa PrPSc backbone (Bessen & Marsh, 1994). Interestingly, following experimental co-infection with both TME strains, Bartz et al. (2000) have demonstrated that either the DY or the HY propagates in recipient hamsters, with some animals showing the co-presence of both strains. Similar findings have been reported in human CJD, where two different PrPSc types, with a molecular mass of 19 and 21 kDa, respectively, have been shown to co-occur in some subjects (Parchi et al., 1999; Puoti et al., 1999). Taken together, the co-occurrence of two different PrPSc types in iatrogenic scrapie suggests that the pool of mammary gland and brain homogenates used for vaccination might have contained two different prion strains.

It is possible, however, that the 20 kDa isoform represents a brain-derived PrPSc type, whereas the isoform that migrates faster corresponds to a peripherally derived PrPSc type. Consistent with the foregoing hypothesis is the finding that the size of the unglycosylated PrPSc fragment migrates faster in lymphoid organs as opposed to brain tissues from sheep with natural scrapie (Madec et al., 2000).

Recently, PrPSc typing, based primarily on the size of the PK-resistant unglycosylated PrPSc fragment, has been performed in contemporary and archival brain tissues from experimental sheep BSE and sheep scrapie (Hope et al., 1999). According to this study, one isolate of natural scrapie, CH1641, with a fragment size of 22-7 kDa (type C pattern), was found to show a migration pattern similar to that seen with experimental sheep BSE. On the contrary, most of the natural sheep scrapie had a type B pattern (20-2 kDa), with a single sheep showing a type A pattern (24 kDa) shared with the SSBP/1 strain. Our findings in Italian natural sheep scrapie and in animals from the infected flock are consistent with a type B pattern, as to the fragment size and the glycosylation pattern of the 20 kDa isoform. Conversely, the 17 kDa PrPSc species is likely distinct from scrapie-associated PrPSc types described previously, with molecular masses ranging from 19 to 24 kDa (Hope et al., 1999, 2000).

To date, no consensus exists on the electrophoretic PrPSc pattern of experimental BSE-infected sheep, which has been reported as being both similar (Hope et al., 1999; Baron et al., 2000) and different (Hill et al., 1998) from the CH1641 scrapie isolate. To overcome the limitations of conventional molecular PrPSc typing, we propose a combined approach that includes, in addition to analysis of fragment length, the conformational stability of PrPSc at acidic and basic pH.

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