JOURNAL OF VIROLOGY, Jan. 2011, p. 1136–1139 Vol. 85, No. 2
0022-538X/11/$12.00 doi:10.1128/JVI.02022-10

NOTES

Sheep with Scrapie and Mastitis Transmit Infectious Prions through the Milk▽

Ciriaco Ligios,[1]† Maria Giovanna Cancedda,[1] Antonello Carta,[2] Cinzia Santucciu,[1] Caterina Maestrale,[1] Francesca Demontis,[1] Mariangela Saba,[1] Cristiana Patta,[1] James C. DeMartini,[3] Adriano Aguzzi,[4]†* and Christina J. Sigurdson[4,5,6]†*

Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy[1]; Research Unit of Genetics and Biotechnology, DIRPA, AGRIS Sardinia, Olmedo, Italy[2]; Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado[3]; Institute of Neuropathology, UniversitätsSpital Zürich, Zürich, Switzerland[4]; Department of Pathology, School of Medicine, University of California, San Diego, California[5]; and Department of Pathology, Microbiology, and Immunology, University of California, Davis, California[6]

Received 23 September 2010/Accepted 5 November 2010

Prions are misfolded proteins that are infectious and naturally transmitted, causing a fatal neurological disease in humans and animals. Prion shedding routes have been shown to be modified by inflammation in excretory organs, such as the kidney. Here, we show that sheep with scrapie and lentiviral mastitis secrete prions into the milk and infect nearly 90% of naïve suckling lambs. Thus, lentiviruses may enhance prion transmission, conceivably sustaining prion infections in flocks for generations. This study also indicates a risk of prion spread to sheep and potentially to other animals through dietary exposure to pooled sheep milk or milk products.

Prion diseases have emerged globally as a significant threat to human and animal health. Recently, human-to-human spread of prions is believed to have occurred through blood transfusions (9, 12, 16), underscoring the importance of understanding possible transmission routes. PrP^{Sc}, a misfolded, aggregated form of the normal prion protein, PrP^{C}, commonly accumulates in the follicles of lymphoid tissues, prior to entering the central nervous system (2, 11, 14). Inflammation can cause lymphoid follicles to form in other organs, such as liver and kidney, which leads to prion invasion of organs that are not typically prion permissive (1). In mice, prion infection in the inflamed kidney has the untoward consequence of prion excretion in urine (13). This finding, together with our report of sheep with PrP^{Sc} in the inflamed mammary gland (8), has raised concerns of prion secretion into milk.

Here, we investigated whether PrP^{Sc} in the inflamed mammary gland leads to prion secretion in milk and infection of naïve lambs through suckling. Prion infectivity has been detected in the milk of sheep expressing a prion gene (*Prnp*) coding for VRQ/VRQ or VRQ/ARQ at polymorphic codons 136, 154, and 171 (3, 4). However, whether (i) sheep-to-lamb transmission of prions in milk leads to clinical prion disease or (ii) sheep with the common ARQ/ARQ *Prnp* genotype can infect lambs through milk is unknown. We induced a chronic lentiviral mastitis and inoculated ARQ/ARQ Sarda breed sheep with infectious prions. After 14 months, we bred the sheep and collected the milk. To avoid cross-contamination of newborn lambs, we fed the milk to imported known-naïve lambs and then monitored the lambs for signs of prion infection (Fig. 1A).

To induce a chronic lymphofollicular mastitis, we exposed 7- to 10-day-old lambs (groups of 10) by intratracheal and intravenous routes to a common sheep lentivirus known as maedivisna virus (MVV) or to cell culture medium only. To do this, lambs were inoculated with 3.5 ml intravenously and 0.5 ml intratracheally of MVV in culture supernatant containing 1.5×10^6 tissue culture infectious doses/ml of the "rapid/high" MVV strain 85/34 (5, 15). Twenty days later, all lambs were orally inoculated with 25 ml of 10% scrapie-infected brain homogenate from a pool of naturally infected Sarda sheep.

We sequenced the entire *Prnp* gene and found that all lambs expressed the ARQ/ARQ *Prnp* genotype, indicating that the sheep should be susceptible to scrapie. As negative controls, 2 lambs of *Prnp* genotype ARR/ARR and ARQ/ARQ were mock inoculated with cell culture medium and healthy brain homogenate. All lambs originated from scrapie-free flocks that had been monitored for clinical scrapie cases for at least 3 years.

All inoculated sheep were naturally bred to rams at 15

* Corresponding author. Mailing address for Christina J. Sigurdson: Department of Pathology, 9500 Gilman Drive, University of California, San Diego, CA 92093-0612. Phone: (858) 534-0978. Fax: (858) 246-0523. E-mail: csigurdson@ucsd.edu. Mailing address for Adriano Aguzzi: UniversitätsSpital Zürich, Institute of Neuropathology, Department of Pathology, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland. Phone: 41 (44) 255-2107. Fax: 41 (44) 255-4402. E-mail: adriano.aguzzi@usz.ch.

† C. Ligios, C. J. Sigurdson, and A. Aguzzi are co-senior authors.

▽ Published ahead of print on 17 November 2010.

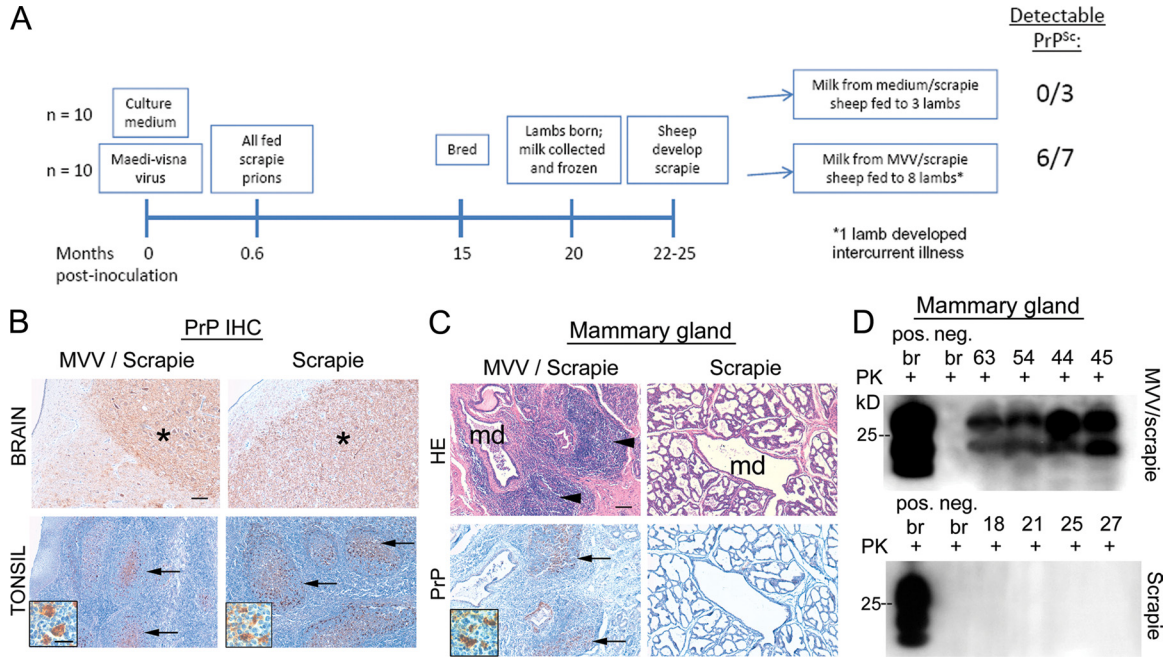

FIG. 1. Sheep infected with prions and maedi-visna virus (MVV) develop lymphofollicular mastitis with PrP^{Sc}. (A) Experimental scheme. Sheep were inoculated with culture medium or MVV and were then orally exposed to scrapie prions and bred. Milk was collected near the time point that neurologic signs of scrapie developed and was fed to naïve lambs. The ratio of lambs with detectable PrP^{Sc} to lambs fed the indicated milk is shown for each experiment. (B) PrP immunohistochemistry assay of brain and tonsil from milk source sheep shows staining for PrP^{Sc} in the brainstem, particularly in the vagal nucleus (indicated by asterisks) and in the tonsillar follicles of scrapie-infected sheep (arrows). (C) Mammary gland (MG) of milk source sheep shows lymphoid follicles (arrowheads) with associated PrP^{Sc} (arrows) adjacent to milk ducts (md) in the MVV-inoculated sheep, whereas the medium-inoculated sheep had a histologically normal MG with no detectable PrP^{Sc}. Insets show a high magnification of follicles containing PrP^{Sc}. Scale bar = 100 μm; scale bar in inset = 25 μm. (D) Western blot analysis shows PrP^{Sc} detection in MG of sheep inoculated with MVV/scrapie agents but not in sheep inoculated with scrapie prions only. The sheep identification number is indicated for each lane. PK, proteinase K digested; pos. br, positive brain control; neg. br, negative brain control.

months postinoculation (p.i.) and produced lambs at 20 months p.i. Sheep developed early signs of scrapie just after the lambs were born. Milk from each sheep was manually collected and frozen daily.

Eight of 10 MVV-and-scrapie (denoted MVV/scrapie)-inoculated sheep and 9 of 10 scrapie-inoculated sheep showed clinical signs of scrapie, with mean incubation periods of 22 ± 1.4 and 23 ± 1.5 months postinoculation, respectively, and were euthanized. There was no significant difference in incubation period between the groups (Student's t test, $P = 0.5$), indicating that inflammation associated with the MVV infection does not accelerate prion disease. This finding is consistent with the results of previous studies that showed that chronic pancreatitis or nephritis did not affect the scrapie incubation period (1). Scrapie infection was confirmed postmortem by the detection of PrP^{Sc} in brain and lymphoid tissues by Western blot and immunohistochemistry assays (Fig. 1B). Interestingly, scrapie did not develop in 3 sheep with a *Prnp* gene

TABLE 1. Genotype, breed, and PrP^{Sc} detection in lambs fed milk from MVV/scrapie- or scrapie-infected sheep

Lamb (dimorphism[a])	Milk source infected with:	Amt of milk ingested (liters)	Breed	Clinical signs present	PrP^{Sc} detected by WB/IHC in:		Time point postinoculation (mo)
					Brain	Tonsil	
951	MVV/Scrapie	1.2	Cheviot	No	−/−	−/−	12
326 (127G/V)	MVV/Scrapie	1.9	Sarda	No	−/−	−/−	28
328 (127G/V)	MVV/Scrapie	1.8	Sarda	Yes	+/+	+/+	28
327	MVV/Scrapie	1.4	Sarda	Yes	+/+	+/+	25
847	MVV/Scrapie	1.3	Cheviot	Yes	+/+	+/+	23
329	MVV/Scrapie	2.1	Sarda	Yes	+/+	+/+	25
843 (141F/L)	MVV/Scrapie	1.3	Cheviot	No	+/+	+/+	28
849 (141F/L)	MVV/Scrapie	1.8	Cheviot	No	+/+	+/+	29
953 (141F/L)	Scrapie	1.5	Cheviot	No	−/−	−/−	28
956 (141F/L)	Scrapie	1.7	Cheviot	No	−/−	−/−	28
957 (141F/L)	Scrapie	1.4	Cheviot	No	−/−	−/−	28

[a] The *Prnp* genotype of all lambs was ARQ/ARQ at codons 136, 154, and 171. Additional dimorphisms in other codons of *Prnp* are noted.

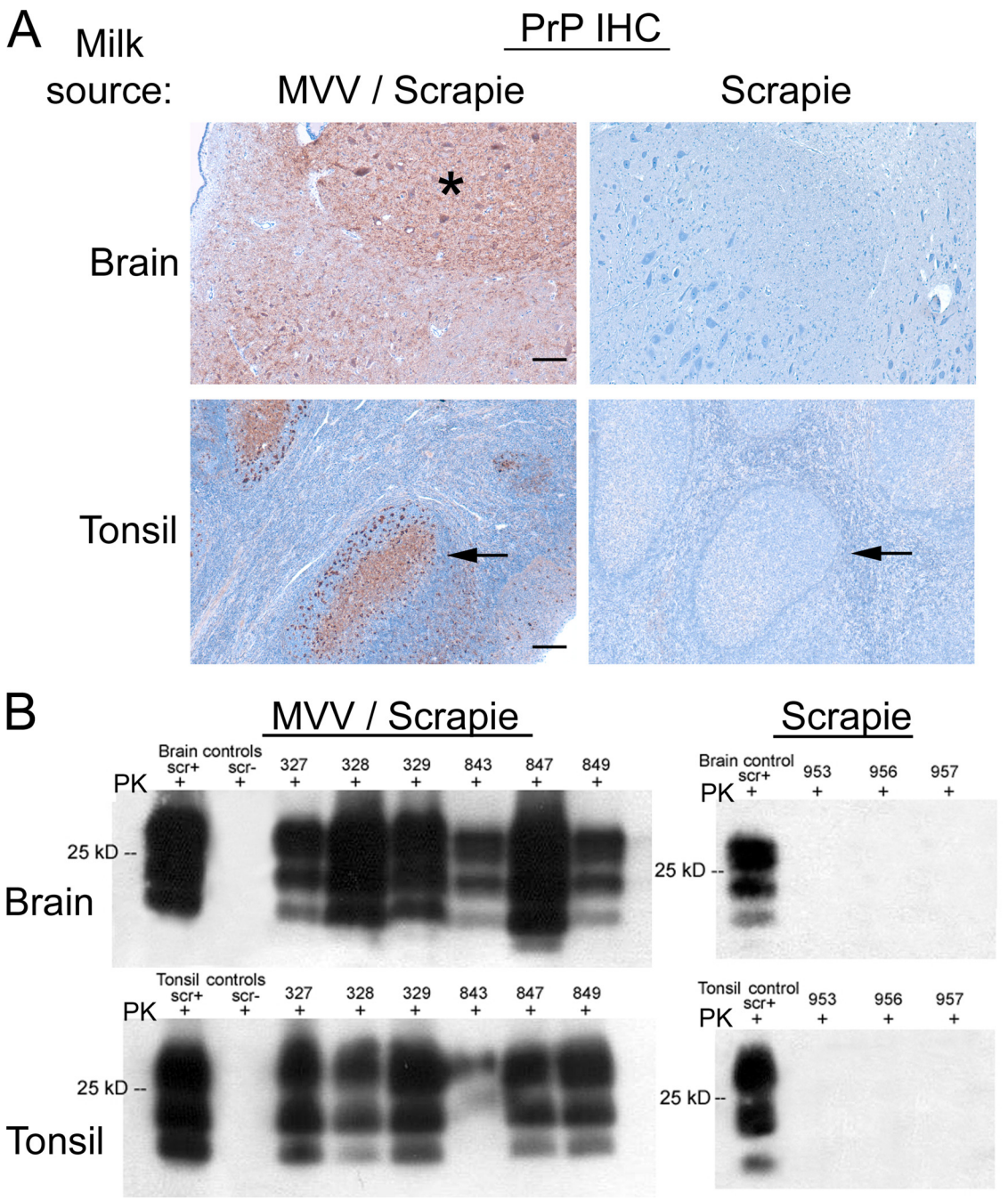

FIG. 2. Lambs developed prion infection through suckling milk from scrapie-infected sheep with mastitis. Brainstem and tonsil from lambs ingesting milk from MVV/scrapie- or scrapie-infected sheep were immunostained for PrP (A) or proteinase K digested (PK) and examined by Western blotting (B). The results show that only the lambs suckling the milk derived from MVV/scrapie-infected sheep accumulated PrP^{Sc}. The sheep identification number is indicated for each lane. scr+, scrapie-positive control; scr−, scrapie-negative control. Scale bars = 100 μm.

encoding a rare polymorphism at codon 176 (K), consistent with recent reports describing scrapie resistance for this genotype (10).

Antibodies to MVV were detected in serum of all the MVV-inoculated sheep by indirect enzyme-linked immunosorbent assay (ELISA) (Elitest kit; Hyphen BioMed). Five of 8 MVV/scrapie-infected sheep (63%) showed a lymphofollicular mastitis (Fig. 1C), and 3 had a diffuse interacinar lymphoid infiltrate. Of the 5 sheep with lymphofollicular mastitis, 4 had PrP^{Sc} deposits detectable by immunohistochemistry and Western blot assays (Fig. 1C and D), whereas no sheep with diffuse lymphoid infiltrates had detectable PrP^{Sc}. Surprisingly, 2 of 9 sheep inoculated only with scrapie also had lymphofollicular mastitis and anti-MVV antibodies, one of which had visible

PrP^{Sc} deposits. MVV is a common pathogen in Europe, and it is possible that these sheep were infected from the dam. The remaining 7 scrapie-inoculated sheep had histologically normal mammary glands (Fig. 1C) and no detectable PrP^{Sc} (Fig. 1D) or anti-MVV antibodies.

We selected the stored milk from the 4 MVV/scrapie-infected sheep with PrP^{Sc} in the mammary glands and from the 7 scrapie-infected sheep with histologically normal mammary glands. Milk samples from the early, middle, and late stages of lactation were pooled for each group. We imported naïve Cheviot lambs ($n = 9$) from flocks that originated from scrapie-free New Zealand and had been bred and housed under strict biosecurity containment in the United Kingdom to ensure that the lambs had not been exposed to scrapie. The Sarda lambs ($n = 4$) originated from a scrapie-free flock in Sardinia. We then fed pooled milk from MVV/scrapie-infected sheep to each of 8 naïve ARQ/ARQ lambs and from scrapie-infected sheep to 3 naïve ARQ/ARQ lambs *ad libitum*. Each lamb ingested a total volume of 1 to 2 liters over a total period of 3 days (Table 1). Two lambs were orally inoculated with brain homogenate pooled from the scrapie-infected milk donors as positive controls. Groups of lambs were housed in separate stalls and subjected to isolation conditions.

Of the 8 lambs fed milk from MVV/scrapie-infected sheep, 1 was sacrificed early and 4 developed clinical signs of scrapie at 23 to 28 months p.i. (Table 1). The 3 remaining MVV/scrapie-exposed lambs and all control lambs were sacrificed between 28 and 29 months p.i. Both lambs orally inoculated with scrapie brain had PrP^{Sc} deposits detectable in the brain. The lamb from the MVV/scrapie group that was sacrificed early (12 months p.i.) had developed an intercurrent illness and had no biochemical or histologic evidence of scrapie infection. However, 6 of the 7 (86%) remaining lambs exposed to milk from the MVV/scrapie-infected dams had detectable PrP^{Sc} in the brain and lymphoid tissues (Fig. 2), indicating that infection from prion-laden milk was dependent on mammary gland inflammation. No lambs fed milk from the scrapie-only infected dams had detectable PrP^{Sc}. We considered that horizontal transmission of prions could have occurred within the MVV/scrapie-exposed lambs; however, Sardinian strains of sheep scrapie are not efficiently transmitted in ARQ/ARQ Sarda sheep, with a maximum recorded prevalence of 41% and an average prevalence of 13% (7).

Previous studies have found that the cellular fraction of milk harbors the most infectivity (4), and the higher leukocyte count in milk that occurs with mastitis could conceivably have increased the infectious prion titers in milk. Our studies in ARQ/ARQ sheep suggest that mammary gland inflammation is necessary for prion transmission through milk, although it remains possible that large milk volumes from sheep without mastitis would transmit prions to nursing lambs. Indeed, milk from VRQ/VRQ sheep without clinical mastitis was previously shown to transmit prion infection to the lambs, as evidenced by PrP^{Sc} deposits in lymphoid tissue biopsy specimens (3).

Taken together, these findings demonstrate that the ingestion of as little as 1 to 2 liters of milk from sheep with scrapie and lymphofollicular mastitis can cause prion infection in ARQ/ARQ lambs at an attack rate of 86%. These data show that a common lentivirus can induce an inflammatory setting highly conducive for prion propagation and secretion in milk, although a role for the virus in transporting prions into the milk or stimulating PrP^{Sc} release from infected cells (6) cannot be excluded. Considering that MVV and other lentiviruses are endemic in sheep and goat populations worldwide, the possibility that lentiviruses have enabled prion transmission through milk and, ultimately, the propagation of scrapie through some flocks should be considered. Together with two other recent reports on infectious prions in sheep milk (3, 4), these studies indicate a risk of prion spread to sheep and, potentially, other animals through dietary exposure to sheep milk or milk products. World milk production contributes up to 13% of the protein supply for humans; thus, studies to determine the extent of infectious prions entering our global food supply would be worthwhile and important for accurate risk assessment.

We are grateful to Hugh Simmons for supplying the Cheviot lambs, Giuseppe Manco for technical support, Nicoletta Ponti for serological data, and Antonio Scala and Valentino Petruzzi for assistance in organizing the experiments. We thank Michael Oldstone for discussions.

This study was supported by The Department for Environment, Food and Rural Affairs, United Kingdom (DEFRA) SE1857, the European Union (TSEUR to A.A. and C.L.), the Novartis Research Foundation (A.A.), the Swiss National Science Foundation, NIH grant no. R21 NS0552116 (C.J.S.), the U.S. National Prion Research Program (C.J.S. and A.A.), and the Italian Ministry of Health (C.L.).

Animal studies were performed in accordance with Italian legislation on animal experimentation (D.L. 116/92).

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