PrPSc Is Not Detected in Peripheral Blood Leukocytes of Scrapie-Infected Sheep: Determining the Limit of Sensitivity by Immunohistochemistry

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Peripheral blood leukocytes (PBLs) from scrapie-infected sheep were evaluated for the presence of PrPSc by using dissociated retropharyngeal lymph node (DRLN) cells and immunohistochemistry (IHC). PrPSc-positive cells were detected in 2.05% ± 0.28% of 3 \times 10^6 DRLN cells, but were not detected in 3 \times 10^6 PBLs from scrapie-infected sheeps. Titration of DRLN cells mixed with PBLs showed that IHC detects a minimum of 0.00205% or 60 PrPSc-positive cells in 3 \times 10^6 PBLs.

Natural sheep scrapie is part of a group of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs), or prion diseases, and is characterized by the accumulation of a protease-resistant protein designated PrPSc. Since PrPSc and a factor associated with infectivity copurify, the presence of PrPSc is considered a marker for TSEs (1).

Immunohistochemical detection of PrPSc is a standard diagnostic method for sheep scrapie. By immunohistochemistry (IHC), PrPSc is detected in lymphoid tissues during preclinical and clinical disease (6, 10, 11, 13, 14). Mouse bioassays correlate with IHC PrPSc detection in lymphoid tissues, where lymph node homogenates from scrapie-infected sheep injected intracerebrally into mice induce scrapie disease (5). However, blood clot or serum from scrapie-infected sheep injected intracerebrally into mice does not induce scrapie disease (5).

Previously, PrPSc was detected in macrophages of dissociated retropharyngeal and prescapular lymph node (DRLN and DPLN, respectively) cells from scrapie-infected sheep by dual IHC (L. M. Herrmann, W. P. Cheevers, W. C. Davis, D. P. Knowles, and K. I. O’Rourke, submitted for publication). However, peripheral blood leukocytes (PBLs) have not been analyzed for PrPSc by IHC. Since a blood-based scrapie diagnostic test would greatly aid live sheep scrapie diagnosis, we evaluated PBLs from scrapie-infected sheep for the presence of PrPSc by using a current diagnostic test for scrapie, IHC. In addition, by using DRLN cells and IHC, the limit of sensitivity of PrPSc detection in PBLs was determined.

Animals. Normal U.S. Suffolk sheep were defined by the absence of PrPSc in the lymphoid tissue of the third eyelid, lymph nodes, and brain by hydrated autoclaving procedures described previously (11). Scrapie-infected U.S. Suffolk sheep were defined as sheep experiencing clinical signs of scrapie at the time of euthanasia and containing PrPSc accumulation in the lymphoid tissue of the third eyelid, lymph nodes, and brain by hydrated autoclaving procedures described previously (11).

Normal and scrapie-infected Suffolk sheep were genotyped as QO at position 171 in the PrP amino acid sequence.

Cells. PBLs were isolated as previously described (7). DRLNs were derived by mechanical disruption of lymph nodes. Mechanical disruption consisted of placing lymph node tissue in a 1.5-ml sterile microcentrifuge tube and punching with a 1-cm2 syringe plunger. Dissociated lymph node (DLN) cells were filtered with a 70-μm-pore-diameter Falcon filter. Filtered cells were centrifuged at 1,500 × g for 10 min at 4°C. The filtered DLN cells were suspended in phosphate-buffered saline (PBS)–10 mM EDTA, and 3 volumes of erythrocyte lysis solution (Gentra) was added. The mixture was incubated for 5 min at room temperature and centrifuged at 500 × g for 10 min at 4°C. The filtered DLN cells were suspended in 5 to 10 ml of wash buffer (PBS [pH 7.2], 10% acid citrate dextrose, 0.1% NaN3, 2% gamma globulin-free horse serum, 1% phenol red), centrifuged at 500 × g for 5 min at 4°C, and counted in 0.4% trypan blue. For cell dilutions, 3 \times 10^2, 3 \times 10^3, and 3 \times 10^4 DRLN cells were mixed with 3 \times 10^6 PBLs. PBLs and DLN cells were fixed in 10% buffered formalin for more than 24 h.

Automated IHC. For PrPSc-positive cell counting, 10% formalin-fixed cells were placed in an area of 1.5 by 1.5 cm (2.25 cm²) on a positively charged glass slide (Superfrost; Fisher Scientific) and air dried overnight. Hydrolytic autoclaving and automated IHC were performed as described previously by using the previously characterized anti-PrP peptide monoclonal antibody (Mab) 99/97.6.1 at 10 μg/ml (11). Negative control antibody (Ventana) raised to a mouse myeloma protein was used as a negative control antibody at 10 μg/ml. Positive cells were defined as having distinct granular cytoplasmic immunoreactivity in cells with a size equal to or larger than that of small lymphocytes. PrPSc-positive cells were counted four times by two investigators (L.M.H. and T.V.B.) in 10 random areas (180 by 180 μm) at a magnification of ×60 with an ocular grid (10 by 10 mm or 1 cm²) and cytometer. The number of PrPSc-positive cells in an area 180 by 180 μm in the four sheep ranged from 6 to 12. The average number of PrPSc-positive cells in an area 180 by 180 μm was calculated for each sheep. Since the cells appeared uniformly distributed on the slide and the average number of PrPSc-positive cells in an area 180 by

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180 μm was known, the average number of PrPSc-positive cells in 2.25 cm² (x) was calculated for each sheep by the formula:

\[ x = \frac{(2.25 \text{ cm}^2 \times \text{average no. of PrPSc-positive cells in 180 by 180 μm})}{(180 \text{ by 180 μm}) \times (1 \text{ cm}^2/10^6 \text{ μm}^2)}. \]

The percentage of PrPSc-positive cells was calculated for each sheep by the formula:

\[ y = \left( \frac{x}{3 \times 10^6} \right) \times 100. \]

PrPSc detection in DRLN cells, but not PBLs from scrapie-infected sheep by IHC. IHC was performed to establish the presence of PrPSc in DRLN cells in scrapie-infected sheep. Using hydrolytic autoclaving and the PrP-specific MAb 99/97.6.1 (11), PrPSc was detected in DRLN cells of four scrapie-infected sheep by IHC (Fig. 1A to D). The percentage of PrPSc-positive cells in 3 × 10⁶ DRLN cells ranged from 1.5 to 2.5%, with a mean percentage of 2.05% ± 0.28% for the four scrapie-infected sheep. PrPSc immunoreactivity was also observed on small aggregates in DRLN cells (Fig. 1B). These
aggregates were considered to be the result of mechanical disassociation of PrPSc-positive cells and were not counted. PrPSc immunoreactivity was not observed in DRLN cells from any of the four scrapie-infected sheep when negative control antibody was substituted for the PrP-specific MAb 99/97.6.1. Also, MAb 99/97.6.1 was not immunoreactive to DRLN cells from four normal sheep (data not shown).

Although PrPSc was detected in DRLN cells from four scrapie-infected sheep according to IHC, PrPSc was not detected in 3 × 10^6 PBLs isolated from the same four scrapie-infected sheep under the same conditions (Fig. 1E to H).

Determination of the minimum number of DRLN cells from scrapie-infected sheep required to detect PrPSc by IHC. Based on the percentage of PrPSc-positive cells in DRLN cells (2.05%), 50 DRLN cells are theoretically required to detect 1 PrPSc-positive cell by IHC. To more accurately determine the minimum number of DRLN cells required to detect PrPSc, 10-fold dilutions of DRLN cells from scrapie-infected sheep were mixed with 3 × 10^6 PBLs from normal sheep and examined by IHC (Table 1). Regardless of the individual sheep, PrPSc-positive cells were consistently detected when 3 × 10^5 DRLN cells from scrapie-infected sheep were diluted with 3 × 10^6 PBLs.

**Table 1.** IHC detection of PrPSc-positive cells in DRLN cells from four scrapie-infected sheep mixed with 3 × 10^6 PBLs from a normal sheep

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<thead>
<tr>
<th>No. of DRLN cells added to 3 × 10^6 PBLs</th>
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<tr>
<td></td>
<td>Sheep 927</td>
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<tr>
<td>3 × 10^6</td>
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<tr>
<td>3 × 10^5</td>
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a, +, presence of PrPSc-positive cells in a given number of DRLN cells mixed with 3 × 10^6 PBLs.

PrPSc-positive cells were consistently detected when 3 × 10^5 DRLN cells from scrapie-infected sheep were diluted with 3 × 10^6 PBLs. Western blotting (under our conditions), and IHC is 1,000 times more sensitive than Western blotting for PrPSc detection of cell-associated PrPSc. Since approximately 3 ng or 88 fmol of PK-resistant PrP is required for band visualization on a Western blot (under our conditions), and IHC is 1,000 times more sensitive than Western blotting, IHC detects an estimated 88 amol of PrPSc, or 5.3 × 10^7 PrPSc molecules, in 60 PrPSc-positive DRLN cells out of 3 × 10^6 PBLs. Even with the level of sensitivity of 8.8 × 10^7 PrPSc molecules per PrPSc-positive DRLN cell, PrPSc was not detected in PBLs of scrapie-infected sheep.

There are several possibilities that could explain the lack of PrPSc-positive cells in PBLs from scrapie-infected sheep by IHC. One possibility is that the sensitivity of IHC is inadequate to detect PrPSc in PBLs. However, a mouse bioassay experiment, which is considered the most sensitive assay for detecting scrapie, showed that mice inoculated intracerebrally with serum or blood clot from scrapie-infected sheep did not develop scrapie (5). In addition, mice did not succumb to disease after intercerebral inoculation of serum or blood from scrapie-infected mice (3). However, when increased numbers of mice adapted with the Fukuoka-1 strain of human Gerstmann-Straussler Scheinker disease were studied, a low level of infectious units (IU) of approximately 10 to 100 IU/ml was detected in plasma or buffy coat, whereas 10^6 IU/ml was detected in brain (2). This suggests that increasing the number of mice and quantifying the amount of plasma and buffy coat cells inoculated into mice may help in determining whether plasma and buffy coat from scrapie-infected sheep contain low levels of infectivity. The second possible reason for not detecting PrPSc in PBLs from scrapie-infected sheep is that lymph node macrophages (Herrmann et al., submitted), but not PBLs, accumulate IHC-detectable PrPSc. The fact that blood monocytes differentiate into macrophages and PrPSc is not detected in 3 × 10^6 PBLs suggests that PrPSc-positive lymph node macrophages acquire PrPSc in the lymph node and do not acquire PrPSc as blood monocytes. This possibility is supported by the fact that cells resident in lymph nodes—follicular dendritic cells and macrophages—are the only two identifiable cell types that have been found to acquire PrPSc (8, 9). However, if very low numbers of PrPSc-positive cells are present in PBLs (<60/3 × 10^6 PBLs), sorting large numbers of monocytes/macrophages from large numbers of PBLs by flow cytometry may allow subsequent detection of a few PrPSc-positive cells by IHC. Another possibility for the lack of PrPSc in PBLs is that PrPSc is not cell associated in the peripheral blood. Recently, others have shown the presence of PrPSc bound to plasminogen in plasma of scrapie-infected mice (4). One last possibility for not detecting PrPSc in PBLs is related to the time of euthanasia. It is possible that PrPSc circulates in PBLs at an earlier time during scrapie infection, and since circulating PBLs have a short half-life, it may be difficult to detect PrPSc in PBLs at the appropriate stage of infection.

In contrast to the results reported here, one report indicated that PrPSc could be detected from PBLs of scrapie-infected sheep by laser-assisted capillary gap electrophoresis (LCGE) (12). However, the sensitivity of LCGE was not determined independently with positive PrPSc cell suspensions. Therefore, the sensitivities of IHC and LCGE for detection of PrPSc in PBLs cannot be compared.

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