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PrP^{Sc} 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 PrP^{Sc} by using dissociated retropharyngeal lymph node (DRLN) cells and immunohistochemistry (IHC). PrP^{Sc}-positive cells were detected in 2.05% ± 0.28% of 3×10^6 DRLN cells, but were not detected in 3×10^6 PBLs from scrapie-infected sheep. Titration of DRLN cells mixed with PBLs showed that IHC detects a minimum of 0.00205% or 60 PrP^{Sc}-positive cells in 3×10^6 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 PrP^{Sc}. Since PrP^{Sc} and a factor associated with infectivity copurify, the presence of PrP^{Sc} is considered a marker for TSEs (1).

Immunohistochemical detection of PrP^{Sc} is a standard diagnostic method for sheep scrapie. By immunohistochemistry (IHC), PrP^{Sc} is detected in lymphoid tissues during preclinical and clinical disease (6, 10, 11, 13, 14). Mouse bioassays correlate with IHC PrP^{Sc} 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, PrP^{Sc} 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 PrP^{Sc} 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 PrP^{Sc} by using a current diagnostic test for scrapie, IHC. In addition, by using DRLN cells and IHC, the limit of sensitivity of PrP^{Sc} detection in PBLs was determined.

Animals. Normal U.S. Suffolk sheep were defined by the absence of PrP^{Sc} 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 PrP^{Sc} 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 QQ 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 plunging with a 1-cm^2 syringe plunger. Dissociated lymph node (DLN) cells were filtered with a 70-μm-pore-diameter Falcon filter. Filtered cells were centrifuged at $1,500 \times g$ 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 $500 \times g$ for 10 min at 4°C. DLN cells were suspended in 5 to 10 ml of wash buffer (PBS [pH 7.2], 10% acid citrate dextrose, 0.1% NaN_3, 2% gamma globulin-free horse serum, 1% phenol red), centrifuged at $500 \times g$ for 5 min at 4°C, and counted in 0.4% trypan blue. For cell dilutions, 3×10^2, 3×10^3, and 3×10^4 DRLN cells were mixed with 3×10^6 PBLs. PBLs and DLN cells were fixed in 10% buffered formalin for more than 24 h.

Automated IHC. For PrP^{Sc}-positive cell counting, 10% formalin-fixed cells were placed in an area of 1.5 by 1.5 cm (2.25 cm^2) 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. PrP^{Sc}-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 cm^2) and cytometer. The number of PrP^{Sc}-positive cells in an area 180 by 180 μm in the four sheep ranged from 6 to 12. The average number of PrP^{Sc}-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 PrP^{Sc}-positive cells in an area 180 by

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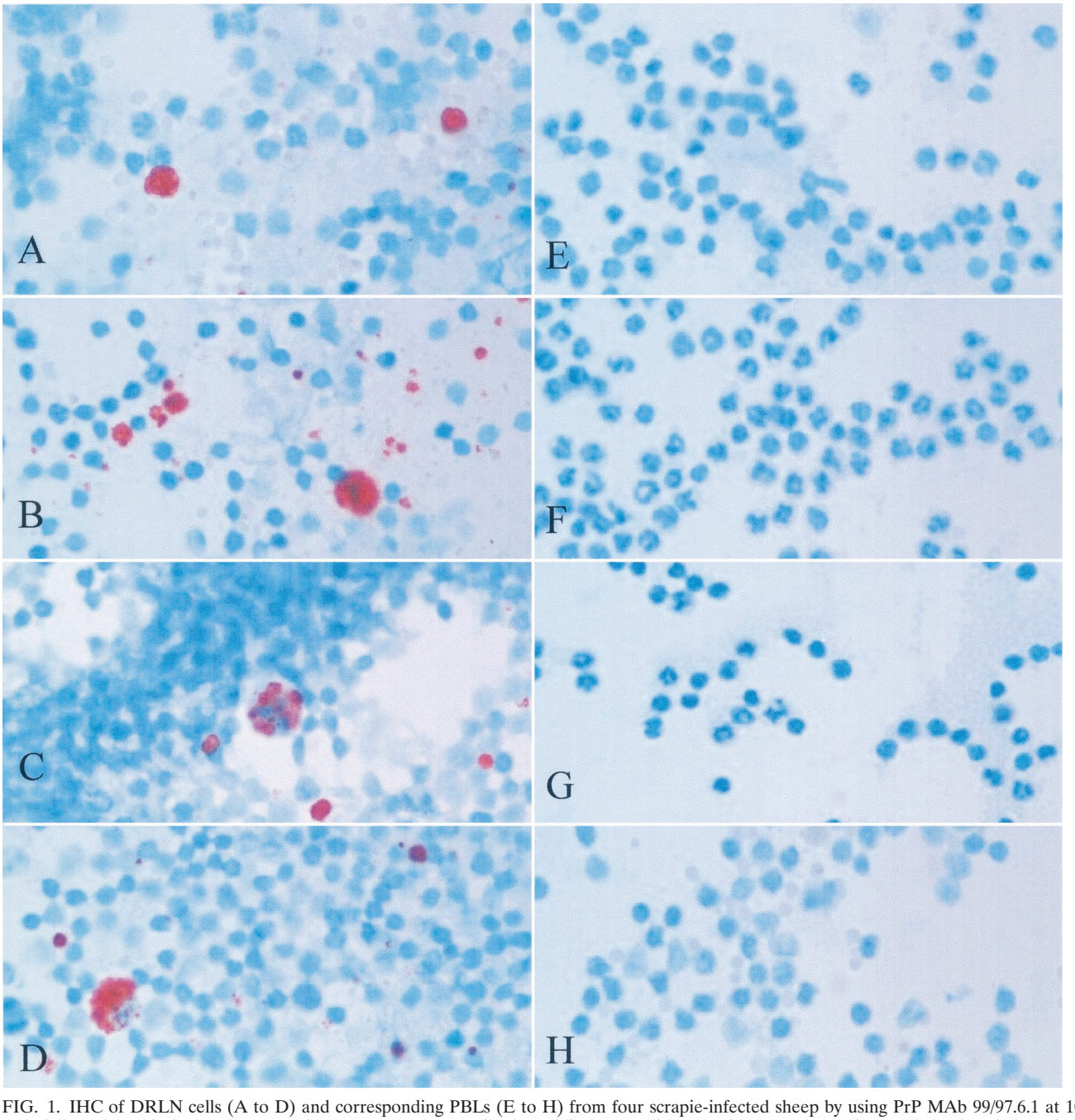

FIG. 1. IHC of DRLN cells (A to D) and corresponding PBLs (E to H) from four scrapie-infected sheep by using PrP MAb 99/97.6.1 at 10 μg/ml, followed by biotinylated goat anti-mouse secondary antibody, avidin-horseradish peroxidase, and 3-amino-9-ethylcarbazoledetection. Magnification, ×1,000.

180 μm was known, the average number of PrP^{Sc}-positive cells in 2.25 cm^2 (x) was calculated for each sheep by the formula x = (2.25 cm^2 · average no. of PrP^{Sc}-positive cells in 180 by 180 μm)/[(180 by 180 μm) · (1 $cm^2/10^8$ μm^2)]. The percentage of PrP^{Sc}-positive cells was calculated for each sheep by the formula $y = [(x/3 \times 10^6) \cdot 100]$. The mean and standard deviation of the percentage of PrP^{Sc}-positive cells from four sheep were calculated.

Prp^{Sc} detection in DRLN cells, but not PBLs from scrapie-infected sheep by IHC. IHC was performed to establish the presence of PrP^{Sc} in DRLN cells in scrapie-infected sheep. By using hydrolytic autoclaving and the PrP-specific MAb 99/97.6.1 (11), PrP^{Sc} was detected in DRLN cells of four scrapie-infected sheep by IHC (Fig. 1A to D). The percentage of PrP^{Sc}-positive cells in 3×10^6 DRLN cells ranged from 1.5 to 2.5%, with a mean percentage of 2.05% ± 0.28% for the four scrapie-infected sheep. PrP^{Sc} immunoreactivity was also observed on small aggregates in DRLN cells (Fig. 1B). These

TABLE 1. IHC detection of PrP^{Sc}-positive cells in DRLN cells from four scrapie-infected sheep mixed with 3×10^6 PBLs from a normal sheep

No. of DRLN cells added to 3×10^6 PBLs	Result for[a]:			
	Sheep 927	Sheep 1243	Sheep 1437	Sheep 1443
3×10^4	ND[b]	+	ND	+
3×10^3	+	+	+	+
3×10^2	+	−	−	−

[a] +, presence of PrP^{Sc}-positive cells in a given number of DRLN cells mixed with 3×10^6 PBLs.
[b] ND, not determined.

aggregates were considered to be the result of mechanical disassociation of PrP^{Sc}-positive cells and were not counted. PrP^{Sc} 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 PrP^{Sc} was detected in DRLN cells from four scrapie-infected sheep according to IHC, PrP^{Sc} 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 Prp^{Sc} by IHC. Based on the percentage of PrP^{Sc}-positive cells in DRLN cells (2.05%), 50 DRLN cells are theoretically required to detect 1 PrP^{Sc}-positive cell by IHC. To more accurately determine the minimum number of DRLN cells required to detect PrP^{Sc}, 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, PrP^{Sc}-positive cells were consistently detected when 3×10^3 DRLN cells from scrapie-infected sheep were diluted with 3×10^6 PBLs.

Comments. According to IHC, the percentage of PrP^{Sc}-positive cells in 3×10^6 DRLN cells from scrapie-infected sheep was $2.05\% \pm 0.24\%$. However, PrP^{Sc} was not detected in 3×10^6 PBLs from scrapie-infected sheep by IHC. A DRLN cell titration revealed that 3×10^3 DRLN cells from scrapie-infected sheep was the minimum number of DRLN cells necessary for PrP^{Sc} detection in 3×10^6 PBLs by IHC. Therefore, the minimum percentage of PrP^{Sc}-positive DRLN cells required for detection in 3×10^6 PBLs is 0.00205%, or 60 PrP^{Sc}-positive DRLN cells in 3×10^6 PBLs. Western blotting with PrP-specific MAb 99/97.6.1 as a primary antibody followed by horseradish peroxidase-labeled goat anti-mouse secondary antibody requires 3×10^6 DRLN cells ($n = 2$) for detection of proteinase K (PK)-resistant PrP^{Sc} (data not shown). Therefore, under our conditions, IHC is 1,000 times more sensitive than Western blotting for PrP^{Sc} detection of cell-associated PrP^{Sc}. 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 PrP^{Sc}, or 5.3×10^7 PrP^{Sc} molecules, in 60 PrP^{Sc}-positive DRLN cells out of 3×10^6 PBLs. Even with the level of sensitivity of 8.8×10^5 PrP^{Sc} molecules per PrP^{Sc}-positive DRLN cell, PrP^{Sc} was not detected in PBLs of scrapie-infected sheep.

There are several possibilities that could explain the lack of PrP^{Sc}-positive cells in PBLs from scrapie-infected sheep by IHC. One possibility is that the sensitivity of IHC is inadequate to detect PrP^{Sc} 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 PrP^{Sc} in PBLs from scrapie-infected sheep is that lymph node macrophages (Herrmann et al., submitted), but not PBLs, accumulate IHC-detectable PrP^{Sc}. The fact that blood monocytes differentiate into macrophages and PrP^{Sc} is not detected in 3×10^6 PBLs suggests that PrP^{Sc}-positive lymph node macrophages acquire PrP^{Sc} in the lymph node and do not acquire PrP^{Sc} 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 PrP^{Sc} (8, 9). However, if very low numbers of PrP^{Sc}-positive cells are present in PBLs ($<60/3 \times 10^6$ PBLs), sorting large numbers of monocytes/macrophages from large numbers of PBLs by flow cytometry may allow subsequent detection of a few PrP^{Sc}-positive cells by IHC. Another possibility for the lack of PrP^{Sc} in PBLs is that PrP^{Sc} is not cell associated in the peripheral blood. Recently, others have shown the presence of PrP^{Sc} bound to plasminogen in plasma of scrapie-infected mice (4). One last possibility for not detecting PrP^{Sc} in PBLs is related to the time of euthanasia. It is possible that PrP^{Sc} 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 PrP^{Sc} in PBLs at the appropriate stage of infection.

In contrast to the results reported here, one report indicated that PrP^{Sc} 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 PrP^{Sc} cell suspensions. Therefore, the sensitivities of IHC and LCGE for detection of PrP^{Sc} in PBLs cannot be compared.

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