CD21-Positive Follicular Dendritic Cells

A Possible Source of PrP^{Sc} in Lymph Node Macrophages of Scrapie-Infected Sheep

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Natural sheep scrapie is a prion disease characterized by the accumulation of PrP^{Sc} in brain and lymphoid tissues. Previous studies suggested that lymph node macrophages and follicular dendritic cells (FDC) accumulate PrP^{Sc}. In this study, lymph nodes were analyzed for the presence of PrP^{Sc} and macrophage or FDC markers using dual immunohistochemistry. A monoclonal antibody (mAb) to the C-terminus of PrP reacted with CD172a+ macrophages and CD21+ FDC processes in secondary follicles. However, a PrP N-terminus-specific mAb reacted with CD21+ FDC processes but not CD172a+ macrophages in secondary follicles. Neither the PrP N-terminus nor C-terminus-specific mAb reacted with CD172a+ macrophages in the medulla. These results indicate that lymph node follicular macrophages acquire PrP^{Sc} by phagocytosis of CD21+ FDC processes. The results also suggest that follicular macrophages have proteases that process full-length PrP^{Sc} to N-terminally truncated PrP^{Sc}. (*Am J Pathol* 2003, 162:1075–1081)

Natural sheep scrapie is part of a group of fatal neurodegenerative diseases termed transmissible spongiform encephalopathies (TSEs) or prion diseases. Sheep scrapie is characterized by the accumulation of a protease-resistant protein designated PrP^{Sc}. Since PrP^{Sc} and the infectious component co-purify, the presence of PrP^{Sc} is considered a marker for TSEs.¹ One predominant theory suggests that PrP^{Sc} is formed by post-translational modification of a normal host-derived protease-sensitive prion protein, PrP^C.^{2,3}

PrP^{Sc} is detected in the brains of scrapie-infected sheep using immunohistochemistry (IHC) with PrP monoclonal antibodies (mAb).^{4–7} In most scrapie-infected sheep, PrP^{Sc} also accumulates in the lymphoid tissues. In addition, lymph node tissue from scrapie-infected sheep

injected intracerebrally into mice induces clinical scrapie.⁸ Spleen, lymph nodes, gastrointestinal-associated lymphoid tissue (GALT), tonsils, and lymphoid tissue of the nictitating membrane or “third eyelid” from scrapie-infected sheep contain PrP^{Sc} in secondary lymphoid follicles.^{9–12}

Some experiments have indicated that there are two PrP^{Sc}-positive cell types in lymphoid tissues of scrapie-infected sheep: tingible body macrophages based on morphology using light microscopy and follicular dendritic cells (FDC) based on morphology and CD21 colocalization with PrP^{Sc} using serial sections.^{13,14} The present study evaluated PrP^{Sc}-positive cell types in lymph node of scrapie-infected sheep by dual IHC using mAb to PrP and macrophage or FDC markers. In addition, mAb to the C-terminus and N-terminus of PrP were used to examine macrophages and FDC for the presence of full-length and truncated PrP^{Sc}.

Materials and Methods

Animals

Normal Suffolk sheep were defined by absence of PrP^{Sc} in the lymphoid tissue of the third eyelid, lymph nodes, and brain using IHC.¹⁵ Scrapie-infected Suffolk sheep were defined as sheep with natural field scrapie experiencing clinical signs and containing PrP^{Sc} in the lymphoid tissue of the third eyelid and brain using IHC.¹⁵ Four normal and scrapie-infected sheep were used in this study. All sheep were homozygous for glutamine at PrP position 171.

Western Blot Analysis

Brain lysates were prepared with and without proteinase K (PK) at 50 µg/ml using a direct tissue homogenate protocol as described.¹⁶ Western blots were performed as described¹⁶ and probed with PrP mAb 99/97.6.1 or 5B2.¹⁶

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Table 1. Flow Cytometric Analysis of Dissociated Lymph Node Cells

mAb	Marker	Mean percentage of positive cells $\pm$ standard deviation	Reference(s)
BAQ15A	CD21	12.63 $\pm$ 10.25	36
BAQ44A	B2	46.98 $\pm$ 13.03	37
BAQ153A	CD11c	5.7 $\pm$ 1.24	38
H58A	MHC Class I	87.58 $\pm$ 11.86	20
H42A	MHC Class II	49.46 $\pm$ 10.86	20
MUC76A	CD11a/18	68.4 $\pm$ 17.37	39
GC1A, 7C2B, GB54A	CD4, CD8, WC1	59.65 $\pm$ 9.99	19
Pig45A2	SIgM	22.14 $\pm$ 13.10	20
8H4	PrP	55.12 $\pm$ 16.65	23
DH59B	MYD1	15.11 $\pm$ 1.49	18–21

Tissue Sections

Frozen sections of prescapular and retropharyngeal lymph nodes to be used for IHC (see below) were cut at 6 μ m on positively charged glass slides (Superfrost, Fisher Scientific), fixed in 10% buffered formalin for 10 minutes, and air dried.

Dissociation of Retropharyngeal and Prescapular Lymph Nodes

Retropharyngeal and prescapular lymph nodes not used for tissue sections were cut in small pieces and placed into sterile Hank's buffered salt solution for mechanical disruption. Lymph node tissue was disrupted in a 1.5-ml sterile microcentrifuge tube using a 1-ml syringe plunger. Dissociated lymph node (DLN) cells were passed through a 70- μ m Falcon filter and centrifuged at 1500 $\times$ g for 10 minutes at 4°C. Filtered DLN cells were suspended in phosphate-buffered saline (PBS), 10 mmol/L ethylene diaminetetraacetic acid (EDTA) and centrifuged at 500 $\times$ g for 10 minutes at 4°C following lysis of red blood cells (RBCs) with three volumes of RBC lysis solution (Gentra). DLN cells were suspended in PBS (pH 7.2), 10% acid citrate dextrose, 0.1% NaN₃, 2% μ -globulin-free horse serum, 1% phenol red, centrifuged at 500 $\times$ g for 5 minutes at 4°C, and counted in 0.4% trypan blue. Aliquots of washed DLN cells were used for flow cytometry or IHC. DLN cells used for IHC were suspended in 10% neutral-buffered formalin for at least 10 minutes, placed on positively charged slides, and air-dried overnight.

Flow Cytometry

DLN cells were analyzed for specific cell surface markers following previous indirect staining protocols.¹⁷ DLN cells were stained with the primary mAbs shown in Table 1. DLN cells were subsequently stained with the following fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated secondary antibodies: FITC-conjugated goat anti-mouse IgM (Caltag) (1:100), FITC-conjugated goat anti-mouse IgG_{2a} (Caltag) (1:100), FITC-conjugated goat anti-mouse IgG_{2b} (Caltag) (1:100), and PE-conjugated goat-anti-mouse IgG1 (Caltag) (1:100). Negative controls included isotype

mAbs substituted for mAbs listed in Table 1 and secondary antibodies used alone without primary antibodies. Cells were resuspended in 200 μ l 1X PBS/2% formaldehyde and analyzed on a FACSsort flow cytometer. A total of 10,000 events were analyzed.

Immunohistochemistry (IHC)

Formalin-fixed tissue sections or DLN cells on positively charged glass were placed into 1X Tris-buffered saline tween (TBST) (Dako) for 5 minutes. Slides were placed into citrate target retrieval buffer pH 6.0 (Dako) and incubated in a pressurized decloaker for 20 minutes at 121°C and 20 psi (decloaking). The slides were cooled 30 minutes and transferred into 1X TBST (Dako) for 5 to 10 minutes. IHC was performed at room temperature. The first IHC used the LSAB2 Kit (Dako), and the second IHC used the second part of the Envision Doublestain kit (Dako) following the manufacturer's instructions. After dual IHC, slides were counterstained with Mayer's hematoxylin (Lillie's Modified, Dako) for 1 minute, washed in Milli Q water, dipped repeatedly in 37 mmol/L ammonium hydroxide, and washed in Milli Q water. Slides were mounted in aqueous mounting solution (Dako) and ex-

Figure 1. Western blot analysis of brain and lymph node from scrapie-infected sheep using mAbs 99/97.6.1 and 5B2. **Lanes 1–3** were detected with 3 μ g/ml of mAb 99/97.6.1 and **lanes 4–6** were detected with 2 μ g/ml of mAb 5B2. **Lanes 1** and **2** contain 200 μ g of brain tissue, and **lanes 4** and **5** contain 800 μ g of brain tissue. **Lanes 3** and **6** contain 2.4 mg of retropharyngeal lymph node tissue. Molecular weights are given on the left.

amed by light microscopy. Digital photomicrography used a Zeiss Axioshop 2 plus microscope equipped with AxioCam imaging software. The following monoclonal antibodies (mAb) were used in the first IHC: DH59B

(IgG₁),^{18–21} GB25A (IgG₁),^{19,22} and TH21A (IgG_{2a}).²⁰ Either PrP C-terminus-specific mAb 99/97.6.1 (epitope at 220–225aa)¹⁵ or PrP N-terminus-specific mAb 5B2 (epitope at 34–52aa)²³ were used in the second IHC. Irrelevant isotype control antibodies coli S 69A (IgG₁) and coli S 205A (IgG_{2a}) (WSU monoclonal antibody lab) were singly substituted for 99/97.6.1, DH59B, GB25A, and TH21A in dual IHC and served as negative controls. Negative reagent controls consisted of using the first mAb in the first IHC sequence followed by all of the dual IHC steps of the second IHC sequence.

Results

Western Blot Analysis of PK-Resistant PrP from Brain and Lymph Nodes of Scrapie-Infected Sheep Using mAbs 99/97.6.1 and 5B2

Both mAb 99/97.6.1 and 5B2 bind to full-length PrP from sheep brain; however, mAb 5B2 does not bind to N-terminally truncated forms of PrP from sheep brain.^{15,23} We confirmed the reactivities of these mAbs by Western blot of brain and lymph node homogenates treated with and without PK. Figure 1 (lanes 1 and 4) shows that both mAb 99/97.6.1 and 5B2 recognized PrP from brain of a scrapie-infected sheep. Following digestion with PK, mAb 99/97.6.1 recognized PK-resistant PrP in brain and lymph node (Figure 1, lanes 2 and 3), whereas mAb 5B2 did not react with PK-resistant PrP in brain and lymph node (Figure 1, lanes 5 and 6). Since the N-terminus of PrP^{Sc} is removed by PK,¹ these results confirm mAb 5B2 recognizes only full-length PrP, whereas mAb 99/97.6.1 recognizes both full-length and N-terminally truncated PrP.

Distribution of PrP^{Sc} in Secondary Follicles of Prescapular Lymph Node Using PrP mAbs

PrP^{Sc} was detected in secondary follicles of prescapular and retropharyngeal lymph nodes of sheep with clinical scrapie using PrP C-terminus-specific mAb 99/97.6.1 or PrP N-terminus-specific mAb 5B2. As reported previously,²⁴ PrP^{Sc} was not detected in lymph nodes of normal sheep using either mAb 99/97.6.1 or mAb 5B2 (not shown). In secondary lymphoid follicles, mAb 99/97.6.1 detected PrP^{Sc} in many macrophage-like cells (defined by nuclear morphology) and to a lesser extent associated with intercellular structures resembling FDC processes. (Figure 2A). However, PrP^{Sc} detected by mAb 5B2 was observed predominantly in FDC processes (Figure 2B). An IgG1 isotype control mAb did not stain secondary follicles (Figure 2C).

PrP^{Sc} and CD172a Co-Localize in Secondary Follicle Macrophages of Prescapular Lymph Nodes Using PrP mAb 99/97.6.1

To confirm the presence of PrP^{Sc} in macrophages of lymph node secondary follicles, dual IHC using a mac-

Figure 2. IHC detection of PrP^{Sc} in secondary follicles of prescapular lymph node sections using two different PrP mAbs. **A:** PrP mAb 99/97.6.1 at 10 μ g/ml (Fast Red). Oil magnification, $\times 63$. **B:** PrP mAb 5B2 at 10 μ g/ml (Fast Red). Oil magnification, $\times 63$. **C:** Isotype control mAb at 15 μ g/ml substituted for PrP mAb 99/97.6.1 (Fast Red). Oil magnification, $\times 63$.

rophage-granulocyte cell-specific mAb (DH59B) and PrP mAb 99/97.6.1 was performed. The mAb DH59B binds to signal regulatory protein α MYD-1 or CD172a on macrophages and granulocytes.¹⁸ Macrophages expressing CD172a were abundant in both secondary follicles and medulla of prescapular lymph nodes (Figure 3A). Using mAb 99/97.6.1 (Fast Red), PrP^{Sc} co-localized with CD172a (DAB) in follicular macrophages (Figure 3B). However, CD172a+ follicular macrophages did not contain PrP^{Sc} recognized by N-terminus-specific mAb 5B2 (Figure 3C). Medullary macrophages expressing CD172a did not contain detectable PrP^{Sc} using either mAb 99/97.6.1 or mAb 5B2 (not shown).

PrP^{Sc} and CD21 Co-Localize in Secondary Follicles of Prescapular Lymph Nodes Using Either PrP mAb 99/97.6.1 or PrP mAb 5B2

FDC are considered a major cell type that accumulates PrP^{Sc} in secondary lymphoid follicles of scrapie-infected mice and sheep.^{13–14,25–29} Since PrP^{Sc} was found in FDC processes (Figure 2A and 2B), dual IHC was performed with CD21 mAb GB25A and PrP mAb 99/97.6.1 or PrP mAb 5B2. Figure 4A shows the distribution of CD21 in secondary follicles of prescapular lymph nodes of scrapie-infected sheep. As expected, FDC processes were positive for CD21 (Figure 4A, arrow 1). CD21 mAb GB25A also stained cells resembling tingible body macrophages (Figure 4A, arrow 2). Tingible body macrophages are defined as macrophages containing bodies in their cytoplasm. As expected from the distribution of PrP staining in Figure 2A, PrP mAb 99/97.6.1 (Fast Red) co-localized with intercellular CD21+ FDC processes (DAB) (Figure 4B, arrow 1) as well as CD21+ macrophage-like cells (Figure 4B, arrow 2; Figure 4C). Similar to PrP C-terminus mAb 99/97.6.1, N-terminus PrP mAb 5B2 co-localized with CD21+ FDC processes (Figure 4D). However, mAb 5B2 did not stain CD21+ tingible body macrophages, supporting the data in Figure 3C indicating that this PrP N-terminus mAb did not stain CD 172a+ macrophages.

IHC Analysis of DLN Cells

The unexpected finding that follicular macrophage-like cells were double-positive for PrP^{Sc} using mAb 99/97.6.1 and CD21 was pursued further using DLN cells to better define membrane boundaries and cell morphology. Flow cytometry of DLN cells from four sheep was used to verify the presence of major lymphoid cell types. Table 1 shows the mAbs used in flow cytometry, the cell surface target of the mAb, and the mean and standard deviation (SD) of the percentage of positive cells for each mAb. These data demonstrate that the major lymphoid cell types were represented in DLN cells.

In DLN cells from scrapie-infected sheep, IHC with PrP mAb 99/97.6.1 detected PrP^{Sc} in macrophage-like cells and membrane debris (not shown). Confirming the lymph node section results in Figure 3B, greater than 95% of

Figure 3. Dual IHC co-localization of PrP^{Sc} and CD172a in secondary follicle macrophages of prescapular lymph node sections of scrapie-infected sheep using a PrP C-terminal mAb 99/97.6.1. **A:** CD172a mAb DH59B at 15 μ g/ml (DAB) with isotype control mAb (15 μ g/ml) substituted for PrP mAb 99/97.6.1 (Fast Red). Magnification of medulla and secondary follicles, $\times 40$. **B:** CD172a mAb DH59B at 15 μ g/ml (DAB) with PrP mAb 99/97.6.1 at 10 μ g/ml (Fast Red). Oil magnification of secondary follicles, $\times 63$. **C:** CD172a mAb DH59B at 15 μ g/ml (DAB) with PrP mAb 5B2 at 10 μ g/ml (Fast Red).

Figure 4. Dual IHC co-localization of PrP^{Sc} and CD21 in FDC processes of secondary follicles of prescapular lymph node sections of scrapie-infected sheep using either a PrP C-terminal-specific mAb 99/97.6.1 or a PrP N-terminal-specific mAb 5B2. **A:** CD21 mAb GB25A at 15 µg/ml (DAB) with isotype control mAb (15 µg/ml) substituted for PrP mAb 99/97.6.1 (Fast Red). Oil magnification, ×63. **Arrow 1** denotes CD21+ FDC processes and **arrow 2** denotes CD21+ cell resembling a tingible body macrophage. **B:** CD21 mAb GB25A at 1.5 µg/ml (DAB) with PrP mAb 99/97.6.1 at 10 µg/ml (Fast Red). Oil magnification, ×63. **Arrow 1** denotes PrP^{Sc} co-localization with intercellular CD21. **Arrow 2** denotes PrP^{Sc} co-localization with intracellular CD21 in a macrophage-like cell. **C:** Digital close-up of **arrow 2** region in Figure 4B. **D:** CD21 mAb GB25A at 1.5 µg/ml (DAB) with PrP mAb 5B2 at 10 µg/ml (Fast Red). Oil magnification, ×63.

PrP^{Sc}-positive macrophage-like cells in DLN cells co-localized with the macrophage marker CD172a. Most of these macrophages were tingible body macrophages (Figure 5A). Consistent with the results in Figures 4B and 4C, PrP^{Sc} also co-localized with intracellular CD21 in many PrP^{Sc}+ tingible body macrophages (Figure 5B). PrP^{Sc}+ macrophages also co-localized with major histocompatibility complex (MHC) class II in >30% of PrP^{Sc}-positive macrophages (Figure 5C).

Discussion

In the present study, PrP^{Sc} and CD21 co-localized in intercellular FDC processes in secondary follicles of lymph node sections from sheep with clinical scrapie using PrP N-terminus-specific mAb 5B2 or PrP C-terminus-specific mAb 99/97.6.1. Following dissociation of lymph nodes, these PrP^{Sc}+ CD21+ processes were recovered as membrane fragments (not shown). Pre-

vious studies by others also indicate that FDC contain PrP^{Sc}.^{13–14,25–29} We also detected both CD 21 and PrP^{Sc} (using mAb 99/97.6.1) in follicular macrophages of lymph node sections as well as macrophages in DLN preparations. These cells were identified definitively by expression of a macrophage marker (CD172a). We attribute the presence of CD21 in follicular macrophages to phagocytosis of FDC, since others have shown that germinal center macrophages acquire antigen-containing FDC processes or iccosomes by phagocytosis.^{30–31} Others have also shown that CD68+ follicular macrophages contain PrP^{Sc} using either a PrP polyclonal Ab R521 (raised to a peptide 94–105aa) or PrP mAb 2G11 (epitope at 151–159aa).³²

Interestingly, we did not detect PrP^{Sc} using the PrP N-terminus mAb 5B2 (epitope 34–52) in follicular macrophages of lymph node sections. One possibility for this observation is that the N-terminus of PrP^{Sc} is removed by macrophage proteases. Thus, PrP^{Sc} in lymphoid follicular

Figure 5. Dual IHC co-localization of PrP^{Sc} using PrP-specific mAb 99/97.6.1 and CD172a, CD21, or MHC Class II in DLN cells from a scrapie-infected sheep. **Panels A–C** were taken at oil magnification $\times 100$ and enlarged digitally. **A:** Tingible body macrophage; CD172a mAb DH59B at 15 $\mu\text{g}/\text{ml}$ (DAB) with 99/97.6.1 at 10 $\mu\text{g}/\text{ml}$ (Fast Red). **B:** Tingible body macrophage; CD21 mAb GB25A at 15 $\mu\text{g}/\text{ml}$ (DAB)/99/97.6.1 at 10 $\mu\text{g}/\text{ml}$ (Fast Red). **C:** Macrophage; MHC Class II mAb TH21A at 15 $\mu\text{g}/\text{ml}$ (DAB)/99/97.6.1 at 10 $\mu\text{g}/\text{ml}$ (Fast Red). Macrophage on **left** shows co-localization whereas macrophage on **right** shows MHC Class II (DAB) staining only.

macrophages may be equivalent to an N-terminally truncated form of PrP^{Sc} commonly found in brain.¹

An important question in the pathogenesis of scrapie is the source of N-terminally truncated PrP^{Sc} in follicular macrophages. The present study indicates that macro-

phages in secondary lymph node follicles of sheep with clinical scrapie acquire PrP^{Sc} by phagocytosis of FDC processes and remove the N-terminus of PrP^{Sc}. Macrophages in the lymph node medulla are another possible source of PrP^{Sc} in follicular macrophages, since secondary follicle macrophages are derived from medullary macrophages.³³ However, CD172a+ macrophages in the lymph node medulla did not contain PrP^{Sc} using PrP mAb 99/97.6.1 or PrP mAb 5B2. Importantly, medullary macrophages were also negative for the FDC CD21 marker. Therefore, the finding that PrP^{Sc} and CD21+ co-localization in macrophages was confined to lymph node secondary follicles demonstrates that follicular macrophages acquired PrP^{Sc} and CD21 exogenously. Since follicular B lymphocytes express CD21,³⁴ tingible body macrophages may acquire CD21 by phagocytosis of B lymphocytes. However, since B lymphocytes are negative for PrP^{Sc},^{32,35} FDC processes are the most likely source of PrP^{Sc} and CD21 in follicular macrophages.

In addition to acquisition of PrP^{Sc} by phagocytosis, macrophages may produce N-terminally truncated PrP^{Sc} constitutively. However, the absence of PrP^{Sc} in lymph node medullary macrophages using the PrP mAb 99/97.6.1 suggests that macrophages do not produce PrP^{Sc}. In addition, PrP^{Sc} is not detected in peripheral blood monocytes of sheep with clinical scrapie using PrP mAb 99/97.6.1.³⁵

In summary, this study presents IHC results identifying PrP^{Sc}+ FDC processes as the probable source of PrP^{Sc} in macrophages of secondary lymph node follicles and that these macrophages remove the N-terminus of PrP^{Sc}. This raises the possibility that FDC may be the only cells in lymphoid tissue which produce full-length PrP^{Sc}. In mice, ME7 strain scrapie pathogenesis is dependent on FDC and not bone marrow-derived cells expressing PrP^C.²⁵ Therefore, FDC expressing PrP^C may be the critical lymphoid cells in sheep scrapie pathogenesis as well. If this is the case, PrP^{Sc} may bind to surface exposed PrP^C on FDC processes, resulting in PrP^C to PrP^{Sc} conversion, and follicular macrophages then acquire PrP^{Sc} by phagocytosis and proteolytically remove the N-terminus from PrP^{Sc}. An important question for future studies is whether follicular lymphoid macrophages participate in the spread of N-terminally truncated PrP^{Sc} to brain.

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