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Small-Ruminant Lentivirus Enhances PrP^{Sc} Accumulation in Cultured Sheep Microglial Cells[▽]

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Sheep scrapie is the prototypical transmissible spongiform encephalopathy (prion disease), which has a fundamental pathogenesis involving conversion of normal cellular prion protein (PrP^{C} [C superscript stands for cellular]) to disease-associated prion protein (PrP^{Sc} [Sc superscript stands for sheep scrapie]). Sheep microglial cell cultures, derived from a *prnp* 136VV/171QQ near-term fetal brain, were developed to study sheep scrapie in the natural host and to investigate potential cofactors in the prion conversion process. Two culture systems, a primary cell culture and a cell line transformed with the large T antigen of simian virus 40, were developed, and both were identified as microglial in origin as indicated by expression of several microglial phenotype markers. Following exposure to PrP^{Sc}, sheep microglial cells demonstrated relatively low levels (transformed cell line) to high levels (primary cell line) of PrP^{Sc} accumulation over time. The accumulated PrP^{Sc} demonstrated protease resistance, an inferred beta-sheet conformation (as determined by a commercial enzyme-linked immunosorbent assay), specific inhibition by anti-PrP antibodies, and was transmissible in a dose-dependent manner. Primary microglia coinfected with a small-ruminant lentivirus (caprine arthritis encephalitis virus-Cork strain) and PrP^{Sc} demonstrated an approximately twofold increase in PrP^{Sc} accumulation compared to that of primary microglia infected with PrP^{Sc} alone. The results demonstrate the in vitro utility of PrP^{Sc}-permissive sheep microglial cells in investigating the biology of natural prion diseases and show that small-ruminant lentiviruses enhance prion conversion in cultured sheep microglia.

Prion diseases (transmissible spongiform encephalopathies [TSEs]) are a group of invariably fatal, transmissible, neurodegenerative diseases, which include scrapie in sheep and goats, bovine spongiform encephalopathy in cattle, chronic wasting disease in deer and elk, and Creutzfeld-Jakob disease and kuru in humans (38). The similarities between scrapie and Creutzfeld-Jakob disease have long been recognized (36), and the use of scrapie as an experimental model allows for the investigation of a natural prion disease in a natural host. The central feature of prion pathogenesis is the conversion of the normal cellular form of the host-encoded prion protein (PrP^{C} [C superscript stands for cellular]) to an abnormal isoform, designated PrP^{Sc} (Sc superscript stands for sheep scrapie) (6, 10, 13). The conversion occurs posttranslationally and involves a conformational change resulting in the generation of a detergent-insoluble, partially protease-resistant molecule that aggregates in affected tissues and serves as the marker for prion diseases. The principal component of the transmissible agent is thought to be the abnormal prion protein and provides the basis for the protein-only hypothesis of prion diseases (50).

There are at least 21 cell lines that have been used to study prion diseases in vitro (59). However, only 4 of these are susceptible to PrP^{Sc} derived from a natural TSE host, while the remaining 17 cell lines are susceptible only to rodent-adapted strains of PrP^{Sc}. Further, only one of the cell lines is derived from a natural TSE host, mule deer (*Odocoileus hemionus*) (51). While rodent-derived cells have many benefits, including the availability of reagents and highly inbred genetics, much of the work accomplished in these cells has to be verified in a natural host-TSE system. The lack of a sheep-derived, scrapie-permissive cell line also prevents full investigation into species-specific phenomena, such as allelic usage variation, allele-predicted susceptibility, and species-specific cofactors. Therefore, the development of a sheep cell culture system would provide an excellent model for such studies.

In addition to creating cell lines that accumulate PrP^{Sc}, it is also desirable to use cells that contribute to the pathophysiology of the clinical disease. Microglia (resident brain macrophages) are such cells, which not only accumulate infectivity in vivo (5) but are also thought to play a role in the neuropathology by their activation and release of immune mediators, such as interleukin-6 (8, 52, 63). Additionally, peripheral macrophages have demonstrated both accumulation (9, 23, 28, 41, 49) and proteolysis of PrP^{Sc} (9, 28). Only one of the current cell culture systems demonstrates microglial or macrophage characteristics, and this cell line is derived from mice and overexpresses the murine prion gene (33). While overexpression of PrP^{C} often increases the permissiveness of cells to PrP^{Sc} accumulation, it can also introduce spontaneous cell pathology (62) and is a confounding factor when trying to study the effects of PrP^{Sc} accumulation at the cellular level.

Another area that would benefit from a natural host-TSE cell culture system is the investigation into possible cofactors for the prion conversion process (55). Identification of these

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accessory molecules is still unresolved; however, several studies suggest that nucleic acids are a possible family of cofactors (2, 14, 15, 18, 19, 21, 64). Interestingly, recombinant prion protein has demonstrated the abilities to bind and to chaperone retroviral RNA, which are similar to retroviral nucleocapsid's function (18, 19, 42). Other interactions between prion protein and retroviruses have been identified; these interactions include an increase in murine leukemia virus (MLV) titers and replication and a shortened scrapie incubation period in the brains of coinfected mice (12, 40). In vitro coinfection studies have also demonstrated increased scrapie infectivity release into the cell culture supernatant in murine cell cultures coinfected with MLV (39).

Sheep, the natural host of scrapie, are also clinically affected by retroviruses, most notably the small-ruminant lentiviruses (SRLVs), visna-maedi virus (VISNA), and caprine arthritis-encephalitis virus (CAEV) (47). These viruses have a worldwide distribution and are the target of eradication programs (47). However, there has been little work published regarding any possible correlation between scrapie and infection with SRLV, with only one report to the authors' knowledge demonstrating a correlation between VISNA-induced lymphofollicular mastitis and resulting PrP^{Sc} accumulation within macrophages and follicular dendritic cells (41). Other studies have demonstrated that chronic inflammation of various organs results in PrP^{Sc} accumulation within those organs that normally lack PrP^{Sc} in prion-affected animals (26, 37), suggesting that the effect of VISNA on PrP^{Sc} in mastitis was indirect. To directly determine whether coinfection with an SRLV increases accumulation of PrP^{Sc}, a sheep microglial cell culture system was developed and utilized in coinfection studies with PrP^{Sc} and SRLV.

MATERIALS AND METHODS

Primary ovine brain cell cultures. Primary mixed glial cell cultures were obtained from an ovine fetal brain using a mechanical dissociation technique for small ruminants previously described in our laboratory (7). The ovine fetus was obtained from a near-term pregnant Suffolk cross ewe that was housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee at Washington State University, Pullman. At approximately day 102 of gestation, the ewe was euthanized by administering an intravenous overdose of barbiturate, and the fetal brain was removed in toto. Approximately 250 mg of brain tissue was removed from the cerebral cortex and used for genotyping of the fetal prion gene as previously described (3). Periventricular white matter tissue from the remainder of the cerebral cortices and midbrain was collected, cut into approximately 5-mm cubes, and disassociated by mechanical triturating in a 25-ml pipette. The resulting brain tissue explants were plated into 75-cm^2 tissue culture flasks with Dulbecco's modified Eagle's medium (Cellgro) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 IU/ml of penicillin, 10 mg/ml streptomycin, and 2.5 μg/ml amphotericin B and left undisturbed for approximately 1 week. Once the cells reached confluence, aliquots of cells were frozen in 90% heat-inactivated FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. Aliquots of cells were thawed as needed and serially passaged, using standard techniques, in OPTI-MEM I reduced serum medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 IU/ml of penicillin, and 10 mg/ml of streptomycin (OMEM).

Transformation of brain cells. Cells were transfected according to the manufacturer's instructions using the LF2000 reagent (Invitrogen), with a plasmid containing the simian virus 40 (SV40) large T antigen, as described previously (25, 27). Primary cells were split in triplicate into 24-well plates and allowed to grow to approximately 60% confluence. Three wells of cells were incubated with plasmid DNA in LF2000 reagent (Invitrogen) and OPTI-MEM for 8 hours at 37°C in 5% CO_2, while the three remaining wells were sham transfected by incubating in LF2000 reagent and OPTI-MEM without plasmid DNA. Following 8 hours, the cell culture medium was changed to OMEM. Cells were fed every 4 days and allowed to grow for 12 days. Cells were then lifted from the plate and passed without dilution into 25-cm^2 plastic tissue culture flasks. Cells were fed every 3 to 5 days as needed and serially passaged 1/10 after reaching confluence. Transfected cells demonstrated an increase in mitotic activity and a loss of contact inhibition. Immunocytochemistry, with the SV40 large-T-antigen-specific monoclonal antibody (MAb) DP02A (Oncogene Research Products, Cambridge, MA), was used to confirm transfection as previously described (27).

Characterization of microglial cell cultures. To characterize brain cell cultures, the brain cell cultures were phenotyped using various cell markers and functional activity assays. The microglial and endothelial marker biotinylated *Ricinus communis* agglutinin-1 (RCA-1) (Dako Cytomation) was used in immunocytochemistry and flow cytometry as previously described (7). Nonspecific esterase activity (Sigma) was performed per the manufacturer's directions (7). Two additional markers, CD14 (catalog no. MM61A; VMRD, Inc.) (immunoglobulin G1 [IgG1]) and CD68 (EBM11; Dako) (IgG1), that are predominately found on cells of the monocytic lineage were tested by immunocytochemistry and flow cytometry. In cells of the monocyte lineage, CD14 is a membrane-bound receptor for lipopolysaccharide (34). CD68 is a lysosome-associated glycoprotein used to identify macrophages (30), and while the MAb EBM11 was raised against human CD68, it has previously demonstrated immunoreactivity against bovine macrophages (1), thus suggesting its utility in this study of sheep cells. For flow cytometric detection of CD14 expression, cells were trypsinized and incubated with the primary antibody. Following three washes, cells were incubated with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody. Cells were then washed twice and fixed in 2% formaldehyde. CD68 is a predominately intracellular antigen; therefore, cells were fixed for 2 days in 10% neutral buffered formalin, permeabilized in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, washed, and then labeled as described above. Five thousand events were analyzed on a FACSort flow cytometer (Becton Dickinson), and counts were determined with Macintosh CellQuest software (BD Biosciences). Results were graphically analyzed using FCS Express (De Novo Software). The Kolmogorov Smirnov test (CellQuest), with a cutoff P value of 0.05, was used to determine significance. Negative controls included the use of isotype-matched antibodies raised against an irrelevant antigen, omission of the primary antibody, and omission of both the primary and secondary antibodies.

For immunocytochemistry, cells were grown in chambered glass slides (Nunc) and allowed to grow to approximately 70% confluence. Cells were rinsed in PBS and fixed in 100% ethanol for 10 minutes. Following quenching of endogenous peroxidase with hydrogen peroxide for 10 min, cells were assayed for expression of the antigens, using the Signet kit (Covance) per the manufacturer's instructions, and the antibodies listed above. The immunolabeling was visualized with 3-amino-9-ethylcarbazole (Dako) with nuclear counterstaining by Mayer's hematoxylin. Negative controls included the use of isotype-matched antibodies raised against an irrelevant antigen, omission of the primary antibody, and omission of both the primary and secondary antibodies.

Inoculation of primary microglia with PrP^{Sc}. Rov9 cells, which are rabbit renal epithelial cells that are stably transfected with the sheep VRQ (Val-136, Arg-154, and Gln-171) allele of the prion gene under the control of a tetracycline-inducible promoter and are susceptible to sheep PrP^{Sc} (60), were used as the inoculum. Rov9 cells with detectable amounts of PrP^{Sc} ($Rov9^{Sc}$) and Rov9 cells that were never exposed to PrP^{Sc} ($Rov9^{C}$) were obtained (B. Caughey with permission from D. Vilette) and maintained in OMEM supplemented with 1 μg/ml doxycycline) as previously described (60). PrP^{Sc} within $Rov9^{Sc}$ cells was verified by PrP^{Sc}-specific immunoblotting and enzyme-linked immunosorbent assay (ELISA) (see below). For use as an inoculum, mechanical lysates of the $Rov9^{Sc}$ and $Rov9^{C}$ cells were prepared as previously described (60). Briefly, the Rov9 cells were grown to confluence in two 75-cm^2 plastic tissue culture flasks. $Rov9^{Sc}$ and $Rov9^{C}$ cells were rinsed three times with sterile 1× Dulbecco's phosphate-buffered saline (D-PBS) and scraped into 10 ml of PBS. The cell pellets were collected by centrifugation at 170 × g at room temperature for 10 min and resuspended in 0.5 ml of filter-sterilized 5% glucose. The cell suspensions were frozen and thawed four times and then subjected to 1 to 2 min of sonication in a cup horn sonicator. The inoculum was stored at −20°C. For inoculation, primary microglia were passed into six-well plates and allowed to grow to approximately 60% confluence. Microglia were rinsed once with PBS and then overlaid with 200 μl of a 1/20 dilution of either the $Rov9^{Sc}$ lysate ($microglia^{Sc}$) or the $Rov9^{C}$ lysate ($microglia^{C}$) in OPTI-MEM. $Microglia^{Sc}$ and $microglia^{C}$ were incubated for 6 hours, and then 200 μl of OMEM was added to each well. Following an additional 2 days of incubation, an additional 0.5 ml of OMEM was added to each well, and microglia were incubated for 4 days at which time they were expanded into 25-cm^2 tissue culture flasks. Microglia were fed

every 3 or 4 days with OMEM as necessary and serially passaged 1/5 after reaching confluence.

Detection of PrP^{Sc} by ELISA. At selected passages following trypsinization, four-fifths of the microglial cell suspension from a 25-cm^2 tissue culture flask was rinsed in D-PBS and then lysed in 120 μl of lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, and 150 mM NaCl) for 3 min at room temperature, followed by centrifugation at approximately 2,300 × *g* at room temperature for 5 min. One hundred microliters of the supernatant was then used for PrP^{Sc} detection by the HerdChek scrapie antigen test kit ELISA (IDEXX) following the manufacturer's instructions. The proprietary ELISA positive and negative controls were used per the manufacturer's directions. A standard curve prepared from diluted $Rov9^{Sc}$ inoculum (1/20, 1/100, and 1/400) was used to normalize corrected optical density results. $Microglia^{Sc}$ results for cells two passages after inoculation with PrP^{Sc} were set at 1, and all other results were normalized to this value. The PrP^{Sc} signal from cells five passages after inoculation with PrP^{Sc} was compared to cells two passages after inoculation using an independent *t* test with a cutoff *P* value of 0.05 (SigmaPlot). Cells were considered positive for PrP^{Sc} accumulation if the corrected optical density was greater than 0.18 plus the negative-control value (per the manufacturer's instructions). To assay for accumulation of PrP^{Sc} over time and to compare the amount of PrP^{Sc} within $microglia^{Sc}$ to the amount in the original inoculum, the PrP^{Sc} signal within late-passage $microglia^{Sc}$ lysates was compared to early passage $microglia^{Sc}$ lysates and to dilutions of the inoculum, respectively, using an independent *t* test with a cutoff *P* value of 0.05 (SigmaPlot). $Microglia^{C}$ lysates served as the experimental negative controls.

Demonstration of PrP^{Sc} infectivity from $microglia^{Sc}$-derived lysates. Inocula were derived from mechanical lysates of $microglia^{Sc}$ and $microglia^{C}$ as described above for the preparation of inocula from Rov9 cells. $Rov9^{C}$ cells (the target cells) were then inoculated as described above with three dilutions (1/20, 1/200, and 1/2,000) of the $microglia^{Sc}$ and $microglia^{C}$ inocula. The Rov9 cells were then expanded once and split 1/5 three times. Lysates from the Rov9 cells were analyzed for PrP^{Sc} accumulation in a single HerdChek scrapie antigen test kit ELISA (IDEXX) plate and reported as the corrected optical density. Cells were considered positive for PrP^{Sc} accumulation if the corrected optical density was greater than 0.18 plus the negative-control value (per the manufacturer's instructions). The PrP^{Sc} signal of each of the $microglia^{Sc}$-exposed Rov9 cells was compared to the signal of the respective $microglia^{C}$-exposed Rov9 cells using an independent *t* test with a cutoff *P* value of 0.05 (SigmaPlot).

Inoculation of immortalized microglia with PrP^{Sc} and antibody-based inhibition. Immortalized microglial cultures were cloned by limiting dilutions in 96-well, plastic, flat-bottom tissue culture plates. Twenty wells contained small colonies that were visualized and split 1/2 into replicate 96-well formats. One replicate was inoculated with 1 μl of the $Rov9^{Sc}$ lysate, and the other replicate was inoculated with 1 μl of the $Rov9^{C}$ lysate (negative control) in 100 μl of OMEM. After the cells reached confluence, the cells and medium (with residual inoculum) were expanded in toto into 24-well plates and cultured in a total volume of approximately 1 ml of OMEM. One week later, an additional 5 μl of $Rov9^{Sc}$ lysate ($Rov9^{C}$ lysate for negative-control cells) was added to the appropriate wells, and 4 days later, 0.5 ml of OMEM was added to each well. When the cells reached confluence, the cells and medium were first expanded into six-well tissue culture plates (approximately 3 ml total of OMEM per well) and then finally into 75-cm^2 tissue culture flasks (25 ml total of OMEM), each time without dilution. Cells were then serially passaged 1/5 at confluence in OMEM using standard techniques. At select passage points, an aliquot of cells was collected, lysed, and evaluated for PrP^{Sc} accumulation by ELISA as described above. Cells were considered positive for PrP^{Sc} accumulation if the corrected optical density was greater than 0.18 plus the negative-control value (per the manufacturer's instructions) after five 1/5 splits or later. PrP^{Sc} was verified by immunoblotting (see below).

PrP^{Sc} accumulation within the PrP^{Sc}-positive SV40 large-T-antigen-transformed cell line (B6) was then inhibited by a prion-specific antibody. Similar to the previously described method, B6 cells were treated for 13 days with the recombinant anti-prion protein Fab D18 (InPro Biotechnology), which has demonstrated the ability to bind membrane-bound PrP^{C} and inhibit PrP^{Sc} accumulation in murine neuroblastoma cells (ScN2a cells) (46). Treated B6 cells were then split 1/5, without antibody, every 4 days for 4 weeks and then collected for immunoblot analysis as described below. The recombinant anti-prion protein Fab R72 (InPro Biotechnology), which does not bind to cell surface PrP^{C} and thus does not inhibit PrP^{Sc} accumulation (46), was used as the negative control. All antibody inhibition experiments were performed in triplicate.

Immunoblot detection of PrP^{Sc} in cell cultures. Since the ELISA does not utilize the standard method of protease digestion to discriminate between PrP^{Sc} and PrP^{C}, cells were also collected for immunoblot confirmation of protease resistance. Primary cells were immunoblotted by a method similar to the method previously described (32). Approximately 2 to 3 million $microglia^{Sc}$ and $microglia^{C}$ were trypsinized into solution and washed with 1× D-PBS. The resulting pellet was lysed for 1 to 2 h in 1 ml of lysis buffer containing 0.5% (vol/vol) Nonidet P-40 (Roche) and 0.5% (wt/vol) sodium deoxycholate (Sigma) in 10 mM Tris buffer (pH 7.4). Aliquots (100 to 250 μl) of lysate were brought up to a total volume of 500 μl by using additional lysis buffer. An equal volume of 4% (wt/vol) *N*-lauroylsarcosine sodium salt (Sigma) solution in PBS was added to the lysate and incubated for 15 min at 37°C, followed by DNase I (100 μg/ml) (Roche) treatment for 30 min at 37°C. Samples were centrifuged 1,100 × *g* for 5 min at room temperature, and 1 ml of the supernatant was treated with proteinase K (50 μg/ml) (Roche) for 1 h at 37°C. Replicate samples were incubated for 1 h at 37°C without proteinase K. Eighty microliters of 4% (wt/vol) phosphotungstic acid in 170 mM $MgCl_2$ was added, the samples were incubated for 1 hour at 37°C, and following centrifugation at 16,500 × *g* at room temperature, the pellet was resuspended in 16 μl of water. Each sample (16 μl) was mixed with 7 μl of 4× NuPAGE sample buffer and 2.5 μl of 10× reducing agent (Invitrogen) and then boiled for 10 min. The samples were electrophoresed for 50 min at 200 V using the NuPAGE precast 12% Bis-Tris-buffered sodium dodecyl sulfate-polyacrylamide gel system (Invitrogen) with morpholinepropanesulfonic acid (MOPS) running buffer. NuPAGE antioxidant (Invitrogen) was added to the inner-chamber running buffer. Gels were electroblotted for 50 min at 300 mA onto a 0.45-μm polyvinylidene fluoride (PVDF) (Millipore) membrane, using the semi-dry Biometra Fastblot B33 apparatus and transfer buffer containing 25 mM Tris (pH 8.3), 150 mM glycine, and 10% (vol/vol) methanol. Following the transfer, the PVDF membrane was fixed for 20 seconds in 100% methanol, air dried, and then immunoblotted or stored dry overnight. PVDF membranes were blocked in casein blocker (Pierce) with 0.05% (vol/vol) Tween 20 for 1 hour at room temperature. The prion protein was detected by a 1-hour incubation at room temperature with the monoclonal antibody F99/97.6.1 (produced in our laboratory; also available from VMRD, Inc.) at a concentration of 3.5 μg/ml in blocking buffer. The membrane was then washed three times in TBST (150 mM NaCl and 0.05% Tween 20 in 10 mM Tris [pH 8.0]) and incubated for 30 minutes at room temperature with a goat anti-mouse IgG1 antibody conjugated to horseradish peroxidase (catalog no. 1070-05; Southern Biotechnology Associates) and diluted 1:5,000 in blocking buffer. Following five additional washes in TBST, the membrane was incubated with chemiluminescent substrate (Western Lightning; Perkin Elmer) for 3 min. Signals were visualized by exposing the membrane to radiographic film (ISC BioExpress) and evaluated for the proper banding pattern associated with proteinase K-resistant PrP^{Sc}.

For detection of PrP^{Sc} within the SV40 large-T-antigen-transformed cell line (B6), the TeSeE Western blot kit (Bio-Rad, France) was used following the manufacturer's instructions. Fifteen to 20 microliters of the final sample suspension was electrophoresed for 60 to 90 min at 250 V using precast 12% Tris-HCl Ready gels (Bio-Rad) in 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% sodium dodecyl sulfate running buffer. Gels were electroblotted for 60 min at 115 V onto a 0.22-μm nitrocellulose membrane (Bio-Rad), using the a Mini-Protean 3 cell (Bio-Rad) and transfer buffer containing 1× Tris-CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] (Bio-Rad) and 15% ethanol. Following the transfer, the nitrocellulose membrane was fixed for 10 s in 100% ethanol and briefly transferred to distilled water. The immunoblotting procedure for the detection of the prion protein was similar to the method described above for the primary microglia, except for the use of reagents, including antibodies, provided in the TeSeE kit.

Inoculation of PrP^{Sc}-accumulating primary microglia with CAEV and evaluation of effect of CAEV on extracellular PrP^{Sc} release. At passage 10 after PrP^{Sc} inoculation, primary $microglia^{Sc}$ were plated at a concentration of 1×10^5/well in 24-well plastic tissue culture plates. $Microglia^{C}$ were treated similarly and served as negative controls for this experiment. The Cork strain of CAEV, in 0.5 ml of total OMEM, was added to the cells ($microglia^{Sc\text{-}CAEV}$ and $microglia^{C\text{-}CAEV}$) at a concentration sufficient to infect the majority of the cells within 7 days (based on a previous viral infection experiment [data not shown]) as assayed by immunocytochemistry, which is described below. Aliquots of both cell types were also sham inoculated with medium only ($microglia^{Sc\text{-}sham}$ and $microglia^{C\text{-}sham}$) to serve as negative controls for the viral inoculation. All inoculations were performed with six replicates. At days 4, 8, 12, 16, and 19 after virus inoculation, culture supernatants were collected and saved at −80°C for analysis by HerdChek scrapie antigen test kit ELISA (see above) and for transmission studies (see below). A standard curve prepared from diluted $Rov9^{Sc}$ inoculum (1/50, 1/100, 1/400, 1/800, and 1/1,600) was used to normalize corrected optical density results between ELISA plates. The PrP^{Sc} signal of $microglia^{Sc\text{-}CAEV}$ supernatants was compared to the PrP^{Sc} signal of $microglia^{Sc\text{-}sham}$ supernatants using an independent *t* test with a cutoff *P* value of 0.05 (SigmaPlot).

TABLE 1. Phenotypes of primary sheep brain cultures and transformed glial cell line

Cells	Method	Marker[a]			
		RCA-I	CD14	CD68	Nonspecific esterase
Primary	Immunocytochemistry	+	+	−	+
	Flow cytometry	+	+	−	NA
Transformed	Immunocytochemistry	+	+	+	+
	Flow cytometry	+	+	+	NA

[a] The presence (+) or absence (−) of markers is shown. The markers are *Ricinus communis* agglutinin-1 (RCA-I), CD14 (catalog no. MM61A; VMRD, Inc.), CD68 (EBM11; Dako), and nonspecific esterase. NA, not applicable.

Demonstration of PrP^{Sc} infectivity from $microglia^{Sc\text{-}CAEV}$-derived supernatants and evaluation of effect of CAEV on intracellular PrP^{Sc} accumulation. Primary $microglia^{C}$ at passage 21 were plated in 24-well plates at 2×10^5 cells per well. One day later, 400 μl of the cell culture supernatant on day 4 after viral inoculation (see previous paragraph) was applied to the cells, in triplicate for each of the four treatment groups ($microglia^{Sc\text{-}CAEV}$, $microglia^{Sc\text{-}sham}$, $microglia^{C\text{-}CAEV}$, and $microglia^{C\text{-}sham}$). Once the cells reached confluence, they were expanded sequentially into 12-well plates and into 25-cm^2 flasks. At day 26 postinoculation, aliquots of microglial cells were plated into chambered slides to confirm that the cells were infected with CAEV (see below), and four-fifths of the total cells were collected and lysed (as described above) for analysis by HerdChek scrapie antigen test kit ELISA. A standard curve prepared from diluted $Rov9^{Sc}$ inoculum (1/100, 1/400, 1/800, and 1/1,600) was used to normalize corrected optical density results. The PrP^{Sc} signal of $microglia^{Sc\text{-}CAEV}$ lysates was compared to the PrP^{Sc} signal of $microglia^{Sc\text{-}Sham}$ lysates using an independent *t* test with a cutoff *P* value of 0.05 (SigmaPlot).

Immunocytochemical detection of CAEV in primary microglia. Cells were fixed with 4% buffered zinc formaldehyde (Z-fix; Anatech Ltd.) for 2 min and then rinsed in 70% ethanol. CAEV antigen was detected using the MAb 10A1 (VMRD, Inc.) directed against 28-kDa capsid protein. Negative controls included the use of isotype-matched antibodies raised against an irrelevant antigen, omission of the primary antibody, and omission of both the primary and secondary antibodies. CAEV-infected goat synovial membrane cells, derived from neonatal goats (29), served as positive controls for the immunocytochemistry.

RESULTS

Establishment and characterization of primary and transformed fetal sheep brain cells. To study the cellular and molecular changes associated with prion conversion in a natural host-TSE system, primary brain cell cultures were derived from a Suffolk cross domestic sheep fetus that was homozygous for the *prnp* VRQ allele (Val-136, Arg-154, and Gln-171). Primary cell cultures were adherent and viable for approximately 20 passages. Additionally, a cell line derived from the primary brain cells was created by transformation with a plasmid containing the SV40 virus large T antigen. Increased rate of growth, lack of contact inhibition, and immunoreactivity for the large T antigen confirmed transformation of the cell culture (data not shown). The transformed cell line has demonstrated viability for 60 passages, as of this writing, and shown no evidence of cell pathology. Both the primary brain cultures and the transformed cell line demonstrated expression of several microglial markers by both immunocytochemistry and flow cytometry (Table 1), although the primary microglia cultures lacked CD68 expression. By immunocytochemistry, approximately 90% of cells demonstrated immunoreactivity for the given markers (data not shown). The approximately 10% of cells lacking immunoreactivity were morphologically indistinguishable from those cells that were demonstrating immunoreactivity. On the basis of the phenotypic features, we classified both the primary cell cultures and the transformed cell line as sheep microglia.

PrP^{Sc} infection of primary microglia. To determine the permissiveness of cultured sheep microglia to PrP^{Sc} infectivity, primary microglia were exposed to the *prnp* genotype-matched $Rov9^{Sc}$-derived PrP^{Sc}. Lysates of the inoculated microglia ($microglia^{Sc}$) were collected at several time points, and commercially available ELISAs were used to screen the lysates for PrP^{Sc}. Primary $microglia^{Sc}$ demonstrated accumulation of PrP^{Sc} over time, as illustrated by an increasing PrP^{Sc} signal in cell lysates, even after dilution of the cells and residual inoculum due to several 1/5 splits (Fig. 1A). The cell growth rate or morphology of $microglia^{Sc}$ and $microglia^{C}$ were not different from each other, which is consistent with previously reported findings (11).

To determine the relative level of PrP^{Sc} accumulation compared to the original inoculum and to further verify the detection of newly converted PrP^{Sc} versus detection of residual inoculum, the amount of PrP^{Sc} in a lysate of microglial cells at passage 9 (representing two expansions and seven 1/5 splits) was compared to the amount of PrP^{Sc} in the inoculum. The inoculum was tested at its original concentration and at a 5^7 (1/78,125) dilution, representing the seven 1/5 splits (and two expansions) of the $microglia^{Sc}$ cells at passage 9. The level of PrP^{Sc} in the $1/5^7$-diluted inoculum was below the detection limit of the ELISA, as evidenced by results similar to those for uninoculated microglia cells. The $microglia^{Sc}$ lysates from passage 9 demonstrated a significantly higher PrP^{Sc} signal than the $1/5^7$-diluted inoculum did (representing the expected PrP^{Sc} signal after seven splits if no accumulation was occurring in the $microglia^{Sc}$) (Fig. 1B). If the cells lacked the ability to form nascent PrP^{Sc}, then the expected result would be that the passage 9 sample would be equal to the $1/5^7$-diluted inoculum. As this was not seen, it was concluded that de novo conversion and accumulation of PrP^{Sc} were occurring within the primary sheep microglia in order to compensate for the dilution of PrP^{Sc} that occurs during splitting of cells during routine cell culture.

The ELISA that was utilized in this study uses a proprietary chemical, which preferentially binds beta-sheets, to distinguish PrP^{Sc} from PrP^{C}. Therefore, immunoblotting of microglial lysates was used to confirm proteinase K resistance, which is the gold standard for PrP^{Sc} detection, of the microglia-derived PrP^{Sc} and to compare the glycoform profile compared to the profile of $Rov9^{Sc}$-based inoculum. $Microglia^{Sc}$ accumulated proteinase K-resistant PrP^{Sc} that migrated with a profile different from that of scrapie-positive brain material (Fig. 2A), but with a profile similar to that of the $Rov9^{Sc}$-based inoculum (Fig. 2B).

The infectivity of the intracellular PrP^{Sc} produced by the microglia was analyzed by exposing $Rov9^{C}$ cells to three dilutions (1/20, 1/200, and 1/2,000) of microglial cell lysates. The $Rov9^{C}$ cells inoculated with $microglia^{Sc}$ lysates demonstrated accumulation of PrP^{Sc} over time as evidenced by an increasing ELISA optical density at each subsequent time point (Fig. 3). Additionally, $Rov9^{C}$ cells exposed to higher concentrations of $microglia^{Sc}$ inoculum demonstrated higher levels of PrP^{Sc} accumulation (Fig. 3). The dose responsiveness of the microglia-derived PrP^{Sc} is consistent with infectivity.

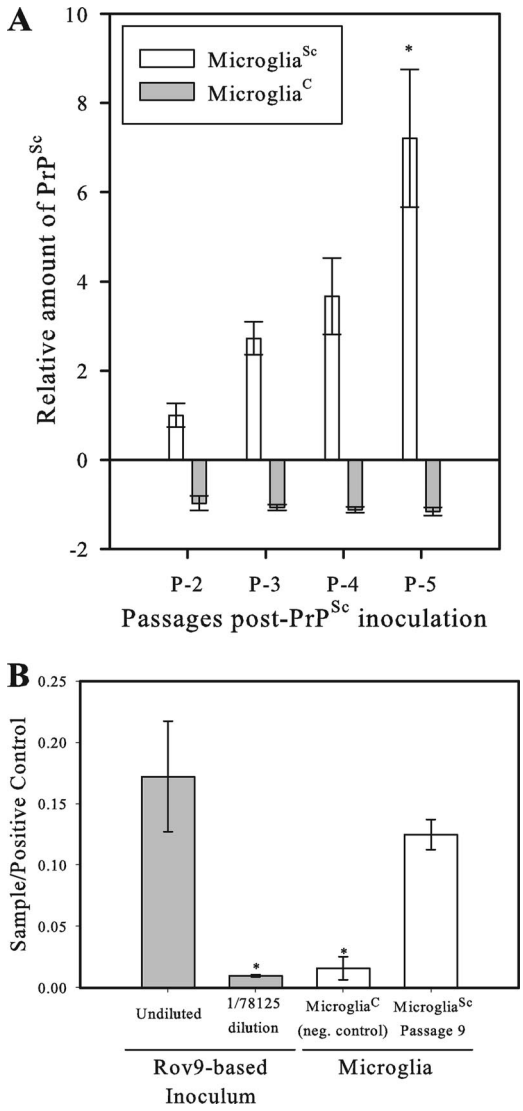

FIG. 1. PrP^{Sc} accumulation in primary microglia. Primary sheep microglia were inoculated in triplicate with either PrP^{Sc}-containing ($microglia^{Sc}$) or PrP^{Sc}-lacking ($microglia^{C}$) cell lysates derived from the PrP^{Sc}-positive or -negative Rov9 cell line (60), respectively, and then serially passaged. Lysates of $microglia^{Sc}$ were assayed for PrP^{Sc} by commercial ELISA, and the corrected optical density was determined. The equation for the corrected optical density is $OD_{450} - OD_{620}$ (OD_{450} being the optical density at 450 nm) per the manufacturer's instructions. (A) ELISA results comparing the level of PrP^{Sc} in late-passage (five passages after PrP^{Sc} inoculation [P-5]) $microglia^{Sc}$ versus early passage (P-2) $microglia^{Sc}$. Data were normalized using a standard curve. $Microglia^{Sc}$ results were set at 1, and all other results were normalized to this value (*y* axis). Each value represents the mean ± 1 standard deviation (error bar). Results for $microglia^{Sc}$ at P-5 and P-2 were compared by a statistical analysis, and the $microglia^{Sc}$ values at P-5 and P-2 were significantly different ($P < 0.005$) as indicated by an asterisk. (B) ELISA results comparing the relative level of PrP^{Sc} in late-passage (P-9) primary $microglia^{Sc}$ to the original inoculum. Data were normalized to the ELISA plate positive control (*y* axis) to account for plate-to-plate variation between testing repeats performed at different times. Results for $microglia^{Sc}$ at passage 9 were compared to the values for $microglia^{C}$ and both $Rov9^{Sc}$-based inoculum data points. The values for P-9 $microglia^{Sc}$ were significantly different ($P < 0.05$) from those for $microglia^{C}$ and the diluted Rov^{Sc} data points, as indicated by an asterisk. neg. control, negative control.

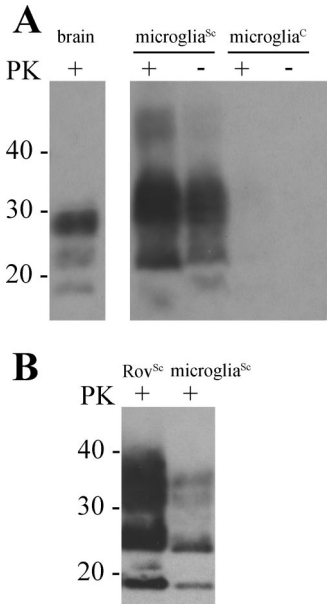

FIG. 2. Immunoblot analysis of primary sheep microglia following exposure to PrP^{Sc} ($microglia^{Sc}$) and unexposed microglia ($microglia^{C}$). Microglia were lysed, treated with proteinase K (PK) (+) or not treated with proteinase K (−), precipitated with phosphotungstic acid, and immunoblotted using the monoclonal anti-PrP antibody F99.97.6.1. (A) Comparison of prion immunoreactivity from sheep brain, $microglia^{Sc}$, and $microglia^{C}$. (B) Comparison of proteinase K-resistant glycoform patterns between $Rov9^{Sc}$ and $microglia^{Sc}$. The positions of molecular mass standards (in kilodaltons) are indicated to the left of the immunoblots.

PrP^{Sc} infection of transformed microglia and inhibition of PrP^{Sc} accumulation. Transformed microglial cells were cloned and inoculated to test whether sheep microglial cell cultures with a longer culture life span than the primary sheep microglial cell culture support PrP^{Sc} accumulation. Clones were inoculated with Rov9-derived PrP^{Sc} and then screened by ELISA for evidence of PrP^{Sc} accumulation. Twenty clones were inoculated, and 15 of those clones were stable and were able to be expanded and tested for PrP^{Sc} accumulation. One of the 15 clones tested positive for PrP^{Sc} by ELISA at passage five and later (data not shown). The positive clone, B6, was the clone that was the slowest to grow to confluence during the initial inoculation period, taking 16 days compared to an average of 6 days for the 14 negative clones. The PrP^{Sc} signal in the transformed cell line remained consistently low, compared to the signal in the primary sheep microglia, and spontaneous loss of PrP^{Sc} was seen after approximately 15 passages.

To further characterize the PrP^{Sc} being accumulated within the transformed microglia, the positive clone was treated with the recombinant anti-prion Fab D18. Following a 13-day exposure to this Fab and 4 weeks of culture without the Fab, the transformed microglial cell line demonstrated loss of PrP^{Sc} detectable by ELISA (data not shown) and detectable by immunoblotting compared to the cultures treated with the negative-control recombinant anti-prion Fab R72, which maintained detectable levels of PrP^{Sc} (Fig. 4). This specific antibody-based inhibition of PrP^{Sc} accumulation and the multiple assays used to detect PrP^{Sc} increase over time indicate

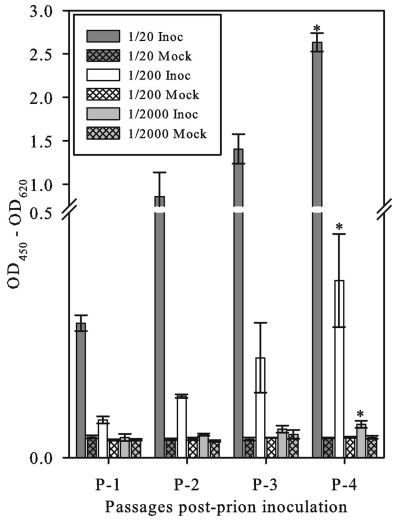

FIG. 3. Transmission of microglia-derived PrP^{Sc} to $Rov9^{C}$ cells. $Rov9^{C}$ cells were inoculated in triplicate with three dilutions of $microglia^{Sc}$ lysates. The inoculated Rov9 cells were serially passaged, lysed, and analyzed for PrP^{Sc} levels by ELISAs. Results for each group of inoculated cells at passage 4 were compared to the results for the corresponding mock-inoculated cells, and the values for inoculated cells at passage 4 that were statistically different ($P < 0.05$) from the value for the corresponding mock-inoculated cells are indicated by an asterisk. All samples were run on the same ELISA plate; thus, results are reported as the corrected optical density (y axis). Each value represents the mean ± 1 standard deviation (error bar) of the sample corrected optical density. The equation for the corrected optical density is $OD_{450} - OD_{620}$ (OD_{450} being the optical density at 450 nm) per the manufacturer's instructions. Inoc, inoculated; Mock, mock inoculated.

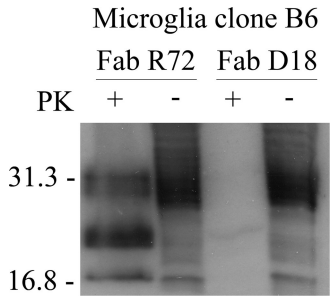

FIG. 4. Immunoblot analysis of PrP^{Sc}-infected sheep microglial cell line after incubation with the recombinant anti-prion Fab D18. Primary sheep cultures were transformed with the SV40 virus large T antigen, cloned by limiting dilutions, and then inoculated with PrP^{Sc}. The clone that was positive for PrP^{Sc} was incubated with Fab D18 for 13 days and then cultured in the absence of the Fab for 4 weeks. Cells were lysed, treated with proteinase K (PK) (+) or not treated with proteinase K (−), and immunoblotted using the TeSeE Western blot kit (Bio-Rad, France). Lysates of replicate PrP^{Sc}-infected B6 cells that were treated with the control anti-prion Fab R72, which does not inhibit PrP^{Sc} accumulation, are included as a control for spontaneous loss of detectable PrP^{Sc}. The positions of molecular mass standards (in kilodaltons) are indicated to the left of the immunoblot.

that the PrP^{Sc} detected in the microglial cells represents PrP^{Sc} molecules that are newly formed in microglia.

Enhancement of PrP^{Sc} levels by CAEV coinfection. Previous studies with ScN2a cells and MLV, a gammaretrovirus, have demonstrated enhancement of prion infectivity release into the culture supernatant (39). While no gammaretroviruses of small ruminants have been identified (17), sheep and goats are susceptible to the SRLVs VISNA and CAEV, which are closely related to one another and are considered members of the same lentivirus group (45). While gammaretroviruses and lentiviruses have unique properties, much of the basic biology between these two groups of retroviruses is shared. To determine whether SRLV coinfection enhances the release of PrP^{Sc} into the culture supernatant, similar to the results of MLV in ScN2a cells, late-passage primary $microglia^{Sc}$ and $microglia^{C}$ were inoculated with the Cork strain of CAEV. CAEV-Cork induced less cytotoxicity than the other tested SRLV strains (VISNA 84-28, VISNA LMH11, and VISNA WLC-1) that each resulted in nearly 100% cell lysis within 1 week (data not shown). The culture supernatants were collected regularly and assayed for relative levels of PrP^{Sc} concentration by normalized ELISA optical densities. There was no detectable difference between cultures infected and uninfected with SRLV in cell growth, as determined by rate to confluence, or phenotype. The results demonstrate that following subsequent infection by CAEV, microglia cells that were already PrP^{Sc} infected have significantly enhanced (up to 1.5-fold) PrP^{Sc} release into the culture supernatant compared to that of $microglia^{Sc\text{-}Sham}$ (Fig. 5A). The supernatants from cultures of $microglia^{C\text{-}CAEV}$ and $microglia^{C\text{-}Sham}$ remained free from detectable levels of PrP^{Sc}, indicating that CAEV does not result in detectable levels of spontaneous conversion of PrP^{C} into PrP^{Sc}.

It is known that the mechanism of PrP^{Sc} transmission within one cell culture system may vary compared to another cell culture system. For instance, in ScN2a cells, the spread of PrP^{Sc} is mainly vertical (i.e., from mother to daughter cells) (22), whereas spread within Rov9 cells is horizontal to nearby cells, with rare spreading to spatially distant cells (44). To determine whether supernatant-derived PrP^{Sc}, which was enhanced by SRLV coinfection in primary sheep microglia, is associated with infectivity toward primary microglia, $microglia^{C}$ were inoculated with supernatants from the previous primary microglia experiment that contained both PrP^{Sc} and CAEV. Additionally, this experiment also served three additional purposes: to repeat the observed enhancement of PrP^{Sc} accumulation following CAEV coinfection, to determine whether the increase in PrP^{Sc} also applied to intracellular PrP^{Sc}, and to determine whether simultaneous PrP^{Sc}-CAEV coinfection (versus CAEV infection of established PrP^{Sc}-infected microglia as shown above) yielded a similar enhancement of relative PrP^{Sc} levels. Lysates were analyzed by ELISA to determine the relative amounts of PrP^{Sc}. Both $microglia^{Sc\text{-}CAEV}$ and $microglia^{Sc\text{-}Sham}$ contain detectable levels of PrP^{Sc}, thus confirming that primary $microglia^{Sc}$-derived PrP^{Sc} is infectious to microglia and suggesting that horizontal spread within microglial cultures is likely (Fig. 5b). Additionally, $microglia^{Sc\text{-}CAEV}$ lysates demonstrated an approximately twofold-higher PrP^{Sc} signal compared to the signal of $microglia^{Sc\text{-}Sham}$ (Fig. 5b). Thus, these results confirm the repeatability of CAEV-induced enhancement of PrP^{Sc} accumulation in microglia, show that the intracellular PrP^{Sc} levels are also elevated, and demonstrate that the PrP^{Sc} enhancement is evident with simultaneous PrP^{Sc} and CAEV coinfections. These two coinfection experiments (Fig. 5) indicate an overall increase, up to twofold, in PrP^{Sc} within primary sheep microglia that are coinfected with CAEV. As expected,

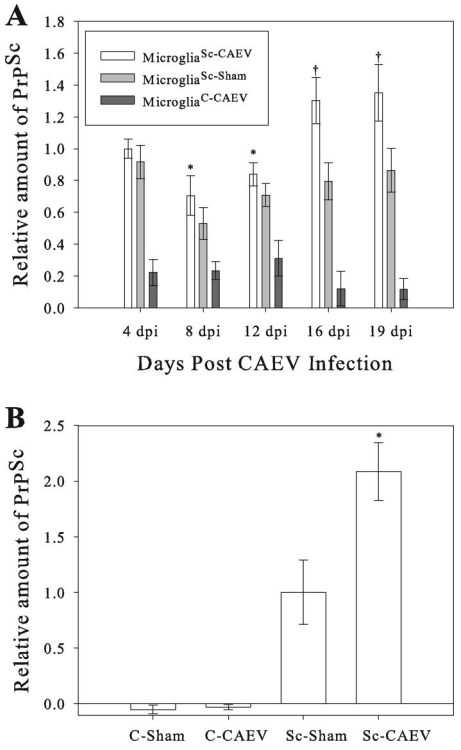

FIG. 5. Effects of CAEV coinfection on PrP^{Sc} accumulation in primary sheep microglia and transmission of microglia-derived PrP^{Sc} to primary microglial cells. (A) PrP^{Sc} accumulation in the culture supernatant of primary microglia infected with CAEV after establishment of PrP^{Sc} accumulation. $Microglia^{Sc}$ and $microglia^{C}$ were inoculated with CAEV, and cell culture supernatants were collected at 4, 8, 12, 16, and 19 days after CAEV inoculation (dpi). Aliquots of supernatant were analyzed by an ELISA for PrP^{Sc} levels, which were normalized via a standard curve. Normalized 4 dpi $microglia^{Sc\text{-}CAEV}$ levels were set at 1, and all other results were normalized based on this value (*y* axis). Six replicates for each treatment group were used. At each time point, results for $microglia^{Sc\text{-}CAEV}$ were compared to the results for $microglia^{Sc\text{-}Sham}$ in a statistical analysis; $microglia^{Sc\text{-}CAEV}$ values that were significantly different from $microglia^{Sc\text{-}Sham}$ values are indicated as follows: ∗, $P < 0.05$; †, $P < 0.0005$. (B) PrP^{Sc} levels in the lysates of primary microglia simultaneously coinfected with PrP^{Sc} and CAEV. $Microglia^{C}$ were inoculated with the 4 dpi culture supernatants from the previous experiment: Sc-CAEV, Sc-Sham, C-CAEV, and C-Sham. At 26 dpi, cell lysates were analyzed by an ELISA for PrP^{Sc} levels, which were normalized via a standard curve. Normalized $microglia^{Sc\text{-}Sham}$ levels were set at 1, and all other results were normalized to this value (*y* axis). Three replicates for each treatment group were used. Results for $microglia^{Sc\text{-}CAEV}$ were compared to the results for $microglia^{Sc\text{-}Sham}$ and evaluated statistically; the $microglia^{Sc\text{-}CAEV}$ value that was significantly different ($P < 0.01$) from the $microglia^{Sc\text{-}Sham}$ value is indicated by an asterisk. Each error bar represents 1 standard deviation.

primary microglia not infected with PrP^{Sc} ($microglia^{C\text{-}CAEV}$ and $microglia^{C\text{-}Sham}$ treatment groups) did not demonstrate detectable levels of PrP^{Sc}. CAEV infection was verified by immunocytochemistry for the CAEV 28-kDa capsid protein (data not shown).

DISCUSSION

Scrapie is an important disease in the United States and has been targeted for eradication (47). While scrapie has been recognized since the early 18th century (48), many questions still remain including the details of transmission between animals and the identification of factors that promote transmission and infection. Recent studies in mice and mouse-derived cell cultures have shown that gammaretroviral infections enhance the amount of prion infectivity produced by prion-infected cells (39). Our results have extended this connection to sheep-derived microglia cultures coinfected with an SRLV. While the VISNA strains that were most readily available were too cytopathic for these studies, we have shown that coinfection with the very closely related SRLV CAEV-Cork strain results in increased PrP^{Sc} accumulation in sheep brain macrophages (microglia), as evidenced by the increase in PrP^{Sc} signal both within the supernatant and within the microglial cells. Additionally, we have demonstrated that the enhancement of PrP^{Sc} accumulation occurs following either sequential PrP^{Sc}/CAEV infection or simultaneous coinfection and that both the intracellular microglia-derived PrP^{Sc} and extracellular microglia-derived PrP^{Sc} are infectious.

Studies analyzing prion and retroviral coinfections in natural hosts are limited. A previous report has demonstrated PrP^{Sc} in macrophages within lesions of SRLV-induced lymphofollicular mastitis in sheep (41). However, the mechanism of colocalization was not determined. One possible mechanism is recruitment of macrophages and follicular dendritic cells, unrelated to prion pathogenesis, to tissues with SRLV-induced inflammation, which then increased the number of PrP^{Sc}-permissive cells in the mammary gland. A second possible mechanism is that the SRLV infection specifically enhances PrP^{Sc} accumulation in the macrophages and follicular dendritic cells. While these two mechanisms are not mutually exclusive, our results demonstrating SRLV-induced enhancement of PrP^{Sc} in microglia (brain-derived macrophages) suggest that a specific interaction may be at least partially responsible for the presence of PrP^{Sc} in SLRV-induced mastitis.

The SLRV-induced enhancement of PrP^{Sc} in these microglia cultures is modest; thus, the in vivo relevance of any direct interaction between SRLV and PrP^{Sc} is not known. However, prion diseases are extremely chronic diseases that take months to years to fully develop (61); thus, small changes in the rate of PrP^{Sc} accumulation can potentially be significant following amplification over that extended time period. Furthermore, both PrP^{Sc} and SRLV are present independently in the same cell types, e.g., macrophages (20, 23, 24, 43, 49), dendritic cells (24, 31, 53), and microglia (5, 7, 16, 33). Additionally, nearly one in four domestic sheep within the United States is infected with VISNA (56). Thus, given the increased PrP^{Sc} accumulation associated with SRLV coinfection that is reported herein, the overlapping cellular tropism of PrP^{Sc} and SRLVs, and the prevalence of SRLV, it is important to determine the extent of interaction between these two agents in vivo and the effects, if any, on any scrapie eradication measures.

This study does not investigate the mechanism of interaction between SRLVs and PrP^{Sc}. Possible mechanisms for the enhanced PrP^{Sc} accumulation in the sheep microglia include but are not limited to the coinfecting SRLV increasing PrP^{C} protein expression, enhancing access to conversion accessory molecules, increasing colocalization of PrP^{C} and PrP^{Sc} during viral assembly, and increasing transmission of PrP^{Sc} by cotransmission with the virus. The study using MLV and ScN2a cells demonstrated that PrP^{C} and PrP^{Sc} are recruited to virions (39),

suggesting that increased PrP^{Sc} transmission is likely to play at least some role in the enhancement of PrP^{Sc} accumulation. Another recent study investigated the interaction of PrP^{Sc} and minute virus of mice, a parvovirus, and demonstrated that the parvovirus-infected cells internalize exogenous PrP^{Sc} more efficiently than the virus-free cells, most likely due to virus-induced changes in the cellular membranes (4). However, it remains to be determined whether similar mechanisms are induced by infections with retroviruses and other viruses. Another possible mechanism for the enhanced prion accumulation is that there are virus-induced changes in the abundance and/or character of RNA, which has been suggested as a possible cofactor for the conversion of PrP^{C} to PrP^{Sc} (2, 15, 19, 21). Determining the mechanism(s) of the virus-induced enhancement of PrP^{Sc} accumulation warrants further study, which may also help to elucidate some of the basic cellular mechanisms of PrP^{Sc} conversion.

During the course of scrapie infection, numerous cell types, including glia, macrophages, and follicular dendritic cells, demonstrate accumulation of PrP^{Sc} (58), but the cell-specific mechanisms that allow for prion conversion are not known. The ability to investigate these mechanisms requires the tools to study PrP^{Sc} infection in these various cell types, mainly having permissive cell culture systems from a variety of cell types. Herein we describe the second cell line derived from a natural TSE host and the first microglial cell culture derived from a natural TSE host (59).

These microglial cells are of the highly susceptible VRQ genotype (Val-136, Arg-154, and Gln-171). Acquiring PrP^{Sc}-containing VRQ/VRQ sheep brain for research material can be difficult in the United States due to the rarity (approximately 0.5%) of this genotype in the United States (57). While it is recognized that this study used an inoculum derived from lagomorph cells, it should be noted that the inoculum's PrP^{Sc} has an ovine, and not lagomorph, amino acid sequence (*prnp* in Rov cells is the ovine insert). Thus, the disadvantage of utilizing material derived from a leporine source is outweighed in this particular case by the availability of Rov9-derived PrP^{Sc} and its matched ovine amino acid sequence. Furthermore, now that the concept of PrP^{Sc}-susceptible sheep microglia cultures has been demonstrated, these cells can be utilized in further studies to determine the relevance of prion allelic usage, prion allelic susceptibility, and other sheep-specific prion questions.

Cell culture models of PrP^{Sc} accumulation have been used for numerous studies to screen for inhibitors of PrP^{Sc} accumulation (59). However, recent work has indicated one of the pitfalls of using cell culture models, namely, that the amount of PrP^{Sc} accumulating in cells is inversely related to the mitotic rate of the cell cultures (22). Thus, the ability to cure cell culture systems of PrP^{Sc} differs significantly compared to mitotically inactive neurons, which are infected with PrP^{Sc} in clinical patients. Our results support this conclusion, as the only positive cell line derived from the transformed microglia was the clone that was slow growing during the initial inoculation phase and even this clone lost the ability to maintain detectable levels of PrP^{Sc} after the cultures began achieving confluence at a faster rate. While the mitotic activity of cell culture systems may alter the biological relevance of using these systems to screen for antiprion compounds, cell culture systems do have the ability to screen for factors that promote PrP^{Sc} accumulation. Multiple lines of evidence suggest a role for an accessory molecule(s) to enable PrP^{Sc} accumulation (35, 54, 55). The coinfection model of PrP^{Sc} and SRLV in primary sheep microglia is one example of using cell cultures to identify factors that enhance PrP^{Sc} accumulation. Further studies, including screening for additional factors that promote PrP^{Sc} accumulation in microglia and studying those cofactors in a variety of PrP^{Sc}-permissive cell lines, are warranted.

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