Spiroplasma found in the eyes of scrapie affected sheep

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Abstract

Objective Scrapie, a transmissible spongiform encephalopathy (TSE) occurring naturally in sheep, characteristically shows a severe retinopathy that is well developed in the terminal phases of the disease. In this study, we set out to demonstrate similar retinal changes in our ruminant spiroplasmosis TSE model.

Procedure The eyes from deer, sheep, and goats that were inoculated intracranially with the laboratory strain of spiroplasma (suckling mouse cataract [SMCA] strain of Spiroplasma mirum) or with Spiroplasma sp. isolated from the brains affected with scrapie or with chronic wasting disease were examined by light microscopy for pathologic changes and by immunocytochemistry for distribution of spiroplasma antigen. The eyes were also obtained from a research flock of sheep with terminal scrapie, from which the intraocular tissues were submitted aseptically for culture assay in M1D broth or as explants on bovine corneal endothelia (BCE).

Results The eyes from the spiroplasmosis ruminant models showed retinopathy remarkably similar to eye lesions seen in sheep with scrapie. The spiroplasma antigen accrued in the ruminant model eye tissues, particularly in the retina, the vitreous humor, and the corneal endothelia. A Spiroplasma sp. grew out of the scrapie-affected eyes both in the M1D broth and in the BCE cultures but did not expand. These new spiroplasma isolates differed immunologically from SMCA.

Conclusion These data showed a clear association of spiroplasma with scrapie suggesting that these bacteria have a role in the pathogenesis of TSE and that the eye should be a research focus for future studies of TSE.

Key Words: prion, retinopathy, scrapie, sheep, spiroplasma

INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) are a group of persistent brain infections of man and animals and include Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, transmissible mink encephalopathy (TME) in farmed mink, and chronic wasting disease (CWD) in deer and elk. All of these diseases are characterized pathologically by widespread vacuolar encephalopathy of the brain gray matter. Scrapie has been experimentally transmitted to rodents by a filterable pathogen approximately 35 nm in size that shows unusual resistance to heat, radiation, and fixatives. A protease-resistant protein marker, called prion, has been found to be deposited in TSE brain and lymphoid tissues during the course of the infection. This miss-folded host protein is detected by Western blots or immunohistochemistry. It is interesting that the scrapie agent is inhibited by tetracycline therapy, which along with evidence that the normal prion isoform may serve as a receptor protein for a bacterium raises the issue whether a conventional organism may also be involved in the pathogenesis of TSE.

This controversy is supported by morphological and molecular studies that suggest spiroplasma, wall-less bacteria known as insect and plant pathogens, are associated with the TSEs. Two strains of Spiroplasma mirum, both rabbit tick isolates, experimentally induce persistent brain infection in mice and in rats with clinical neurological deterioration similar to experimental scrapie in rodents. The spiroplasmosis TSE model in rats has been reproduced by subcutaneous and intraperitoneal inoculation of S. mirum indicating that the microbe is neurotropic. Both the suckling mouse cataract agent (SMCA), a laboratory strain of S. mirum, and Spiroplasma spp., isolated from sheep brains affected with scrapie or from deer brains affected with CWD via passage through embryonated eggs, induce
spongiform brain degeneration in small ruminants. In this study, we have expanded on our prior study to show the occurrence of a unique retinopathy in the spiroplasma infected ruminants that closely resembles the eye lesions found in natural and in experimental scrapie. Spiroplasma antigen was found to be abundant in the eyes of sheep with experimental spiroplasmosis, which then led to experiments wherein a Spiroplasma sp. was isolated from the eyes of sheep with terminal scrapie.

MATERIALS AND METHODS

Animals
The eyes from animals with experimental spiroplasmosis were obtained from a prior study in deer and sheep. Briefly, deer, sheep, and goats were inoculated intracranially (IC) as neonates with the SMCA laboratory strain of S. mirum (a rabbit tick isolate) and Spiroplasma spp. isolated from either scrapie-affected sheep brain or CWD-affected deer brain via passage through embryonated eggs. The results of the pathological examination of the brains of these animals as reported include four deer inoculated IC with SMCA and four control deer (both groups examined at 4.5 months); two sheep inoculated IC with SMCA; and one sheep inoculated IC with M1D growth media (examined at 16–19 months); three sheep and four goats inoculated IC with Spiroplasma spp. derived from scrapie sheep; and one sheep and five goats inoculated IC with Spiroplasma spp. derived from the deer brain affected with CWD with one goat inoculated IC with M1D media (all examined at 11 months). See prior publication for further details.

The eyes (one frozen and one formalin-fixed) from four sheep, three in the terminal stages of scrapie and one clinically normal sheep, were purchased from the Caine Research Center, University of Idaho. Two of the scrapie-affected sheep (#296 and 301) had been orally inoculated with scrapie brain homogenate at 11 months and were euthanized 6 years later. Sheep #5061 developed scrapie by 11 months). See prior publication for further details.

Dark-field microscopy
A 5 μL drop of test sample was placed on a clean glass microscope slide, mounted with a #1.5 cover slip and sealed on all four sides with clear finger nail polish. Slides were examined with a Zeiss Axio Imager A1 microscope (Carl Zeiss, Inc., North America, Thornwood, NY, USA) equipped with a dark-field top lens condenser 1.2–1.4 and an EC Plan-NeoFluar ×100 oil immersion objective (Carl Zeiss, Inc. North America, Thornwood, NY, USA) with a 1.3 iris closed to its lowest setting (0.7).

Histology and immunohistochemistry
The formalin-fixed eyes from deer and sheep with spiroplasmosis and sham-inoculated animals were sectioned horizontally, paraffin-embedded and sections were stained with hematoxylin and eosin (H&E). The adjacent eye sections were immune stained by 3,3′-diaminobenzidine/horse radish peroxidase (DAB/HRP) with antibodies directed against glial fibrillary acidic protein (GFAP) or with hyper-immune rabbit sera directed against SMCA (Courtesy Dr. Maureen Davidson, Purdue University) using the DAKO Sweden automated detection system (Dako North America, Inc., Carpinteria, CA, USA). The formalin-fixed eyes affected with scrapie were sectioned vertically through the tapetum and paraffin sections were stained with H&E.

Direct immunofluorescence assay (IFA)
Bovine corneal endothelia (BCE) (ATCC #CRL-2048) persistently infected with scrapie spiroplasma isolates were sub-cultured onto sterile 12-mm glass cover slips, fixed in cold acetone for 10 min and blocked by flooding with 100 μL of 0.1% Cold Fish Skin Gelatin in ×1 phosphate buffer saline solution (PBSS; pH 7.2–7.4), then incubated in a humidity chamber at 37 °C for 30 min. The cover slips were drained, flooded with 100 μL of fluorescein isothiocyanate (FITC)-labeled rabbit anti-SMCA antibody diluted 1:100 in ×1 PBSS w/v 0.00125% Evans Blue dye, and incubated in a humidity chamber at 37 °C for 30 min followed by rinsing three times with 1 mL of ×1 PBSS. The cover slips were counterstained with 100 μL of 200 μM propidium iodide, incubated in a humidity chamber at 37 °C for 15 min, rinsed as before, air dried and mounted using Vectashield Hard Set (Vector Laboratories, Inc., Burlingame, CA, USA). Slides were viewed with a Zeiss Axio Imager A1 microscope (Carl Zeiss, Inc., North America, Thornwood, NY, USA) equipped with an X-cite 120 fluorescent light source and filters for green and red fluorescence.

Electron microscopy
Centrifuged pellets of trypsinized BCE cultures were fixed in cold 1.25% glutaraldehyde in cacodylate buffer overnight then embedded in epon and sectioned on an ultramicrotome (RMC MT6000 microtome, Boeckeler Instruments, Inc., Tucson, AZ, USA). Thin sections were examined using a Joel JEM 1011 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Molecular analyses
PCR was performed using spiroplasma 16S rDNA-specific primers as per Bastian et al. PCR products excised from agarose gels were sequenced in the Division of Biotechnology and Molecular Medicine, LSU School of Veterinary Medicine.

Direct isolation of spiroplasma in M1D broth and tissue culture
All dissections were performed at BSL2 with IBRDSC approval. The frozen sheep eyes affected with scrapie were thawed in a water bath, and the exterior surface sterilized with 70% alcohol. The aqueous humor was removed using a 25 gauge 5/8 in needle. Each eye component was either
placed directly into 1 mL of M1D broth (a special broth of high osmolarity for culture of spiroplasma and other mollicutes\(^\text{16}\) (whitcomb) or overlaid onto \(\geq 80\%\) confluent BCE cultures. Broth cultures were incubated in closed tubes at 37 °C. Cell cultures with explants were initially incubated at 37 °C for 30 min, after which the full component of media was added and cultures were incubated at 37 °C with 5% CO\(_2\).

RESULTS

Comparison of ocular lesions in experimental spiroplasmosis and scrapie

The eyes from deer and sheep inoculated IC with SMCA showed retinopathy characteristic of natural scrapie.\(^\text{14}\) The pathological changes in spiroplasmosis appeared to involve all layers of the retina extending to the photoreceptor regions as described in experimental scrapie\(^\text{15}\) and shown in Fig. 1b. The depletion and vacuolization of the neuronal cell layers caused marked thinning of the retinas compared with normal controls (Fig. 1a). GFAP immunohistochemistry (DAB/HRP reaction) of the spiroplasma-infected retina revealed presence of GFAP antigen (representing astrogliosis) in the inner regions of the retina (Fig. 2b) as reported in ruminant eyes infected with scrapie\(^\text{17}\) or with the TME agent.\(^\text{18}\) One deer inoculated with spiroplasma showed focal anterior uveitis in addition to retinopathy (data not shown); but for all other spiroplasma infected ruminants, no inflammatory reaction was noted. This unique retinopathy was observed in the eyes from asymptomatic sheep 16 months after IC SMCA inoculation (Fig. 3a).

The eye as a focal point in experimental spiroplasmosis and scrapie

Sparse numbers of SMCA antigens were seen by DAB/HRP immunohistochemistry in the spiroplasmosis sheep retina in close association with vacuolated neurons (Fig. 3a). In addition, large collections of cell-free SMCA antigens were
found in the vitreous humor of a sheep at 16 months post-SMCA IC inoculation without inflammatory response (Fig. 3b), likely representing bacterial extension from the retina into the globe. The endothelia lining the cornea were vacuolated with numerous SMCA antigens on the endothelial surface as well as within the endothelial cells (Fig. 3c). Occasional cell-free spiroplasma antigens were seen in the aqueous humor, although involvement of the anterior chamber of the eye was hard to evaluate as most of the aqueous fluid had washed out during tissue processing (data not shown). Spiroplasma antigens were not seen in corneal stroma, in corneal epithelia, or in peripheral nerve elements in the sclera at the limbic margin.

The abundance of spiroplasma antigen in the eyes of the spiroplasmosis sheep model indicated that the eye was a preferred site for proliferation of the organism. This clue raised the question whether spiroplasma were present in the eyes of scrapie-affected sheep. Several pairs of eyes obtained from a research scrapie flock (Fig. 1d-f) showed retinal pathology. Sheep ID#296 and ID#301 had been exposed to scrapie brain homogenate orally at 11 months, exhibited long subclinical incubation (6 years), and showed severe retinopathy (Fig. 1e, f). Scrapie-affected sheep ID#5061 had developed scrapie through natural transmission at birth with short incubation (2.5 years) and showed less severe retinopathy (Fig. 1d).
All were subjected to culture assay. Aqueous humor, vitreous humor, and corneal tissues were removed aseptically from the frozen eye of scrapie-affected sheep ID#301 and placed in specially prepared M1D media of high osmolality.\textsuperscript{16} After 2 weeks incubation at 37 °C, the M1D media inoculated with the minced corneal tissue became acidic; and spiral forms were found by dark-field microscopy (DFM) repeatedly through several passages over 2.5 months (Fig. 4). M1D media inoculated with other eye components from sheep ID#301 were negative by DFM. Similarly, M1D media preparations inoculated with the eye tissue from the two additional scrapie-affected sheep revealed spiral forms by DFM after 2.5 months of incubation (data not shown). The spiroplasma isolates derived from the scrapie-affected sheep eyes have remained in primary passage stage/nonpropagation stage in the M1D cultures, as determined by observation of two to three spirals by DFM at each time point without evidence of a growth curve. A normal sheep eye and a normal goat eye were processed similarly and were negative for spiroplasma or retinal pathology (data not shown). Spirals were not observed by DFM in control eye preparations or in un-inoculated M1D media.

\textbf{Isolation of scrapie spiroplasma in BCE cell culture}

Vitreous humor from scrapie sheep ID#301 was inoculated onto a mouse N2a neuroblastoma cell culture (In Pro Biotechnology Inc. ICL-N2AI, San Francisco, CA, USA) to enhance the growth of the spiroplasma isolate; and although no spirals were seen in the spent cell-free culture MEM preparations or in un-inoculated M1D media.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{spiroplasma.png}
\caption{\textit{Spiroplasma} sp. isolated from eyes of a research scrapie-affected flock at the University of Idaho. (a–d) These dark-field images at x1200 magnification were taken of a cell-free culture of bacteria obtained from the eye of scrapie-affected sheep ID#301 over several passages. \textit{A Spiroplasma} sp. was isolated from two other eyes obtained from sheep with terminal scrapie (scrapie-affected sheep ID#5061 and ID#296).}
\end{figure}

Figure 4. \textit{Spiroplasma} sp. isolated from eyes of a research scrapie-affected flock at the University of Idaho. (a–d) These dark-field images at x1200 magnification were taken of a cell-free culture of bacteria obtained from the eye of scrapie-affected sheep ID#301 over several passages. \textit{A Spiroplasma} sp. was isolated from two other eyes obtained from sheep with terminal scrapie (scrapie-affected sheep ID#5061 and ID#296).

\textbf{DISCUSSION}

The retinopathy of TSE has been understudied in both animal and human disease. The presence of retinopathy is difficult to assess clinically in sheep affected with scrapie as animals tend to function in spite of significant visual impairment. Scrapie-affected animals in the University of Idaho research flock have been seen to walk into obstructions. Electroretinography has demonstrated retinopathy in experimental scrapie in sheep,\textsuperscript{17} in experimental TME in cattle,\textsuperscript{18} and in experimental scrapie in mice.\textsuperscript{21} Ophthalmic abnormalities occur in a significant number of CJD patients with 10% of cases presenting with blindness and 50% of CJD patients developing ophthalmic deterioration during the course of the illness.\textsuperscript{22,23} Retinopathy has been found in a preclinical CJD patient by electroretinography.\textsuperscript{24} Usually, no ophthalmic studies are carried out on CJD patients as blindness is often considered to be due to cortical pathology.

The distribution of spiroplasma antigens in the spiroplasmosis ruminant model as shown by immunohistochemistry revealed some surprises. Spiroplasma antigen was found in the retina with extension into the globe with large collections involving vitreous and anterior segment structures,
particularly the corneal endothelia. If we consider experi-
mental spiroplasmosis as a TSE model and spiroplasma as a
candidate agent of TSE, this pattern is not that of prion
deposition in the eye in scrapie. Rather, the infection-
related protein is limited to the retina in TSE, although
infectivity is present in the anterior eye segments. It is
important to understand that TSE infectivity and prion are
not necessarily one and the same in that immunoreactivity
does not always correlate with biological activity. If we
extrapolate the findings in our spiroplasmosis model to
TSE, then large concentrations of the infectious agent in the
vitreous humor could explain the reported contamination of
surgical instruments after surgery in the posterior eye of
CJD patients. Persistent infection of the corneal endothel-
ia by the TSE agent may be the mechanism of iatrogenic
transmission of CJD via corneal transplant. The ease of
isolation of Spiroplasma spp. from the eyes of sheep in the
late clinical stages of scrapie would indicate the clear associ-
ation of spiroplasma with TSE and the eye as a preferential
site for detection of the bacterium.

These data presented in this study question proteinase K
resistant prion protein (PrPsc) being the sole factor in the
pathogenesis of TSE especially as prion is not detectable in
5–10% of cases of TSE. Although this discrepancy could be
a sensitivity issue of the Western blot technique, the recent
finding that the prion isoform (PrPc) serves as a receptor
protein suggests otherwise. The interaction between a
bacterium (spiroplasma) and a protein receptor (PrPc) might
well result in conversion of PrPc into abnormally folded
PrPsc. This idea is supported by evidence that interaction of
a ligand with a protein receptor leads to conformational
alteration of the receptor protein resulting in protease resis-
tance. If accumulation of PrPsc causes a toxic effect by
altering cholesterol in the cell membranes, the lesions may
have been induced indirectly by spiroplasma. On the other
hand, the pathology of TSE could be due to the insatiable
appetite of this organism for sphingolipids and choles-
terol. PrPsc deposits have not as yet been shown in the
brain tissues from our spiroplasmosis ruminant models, but
this discrepancy is not alarming as prion has been reported
to be absent when there is experimental cross-species transfer of TSE. In those instances, prion was detected only after serial passage suggesting adaptation of the agent to the new host is necessary before the abnormal protein is deposited in the tissues. Additional studies are underway to determine if our spiroplasmosis model is prion disease or if it just simulates TSE. In either case, spiroplasma is associated with naturally occurring TSE and its role in the pathogenesis must be determined by future investigations.

**SUMMARY**

The isolation of a *Spiroplasma* sp. from the eyes of sheep affected with terminal scrapie strongly supports experimental spiroplasmosis in ruminants as a model for TSE. The aggregation of bacteria in the eyes of the spiroplasmosis ruminant models suggests that future research efforts should focus on the eye for studying the nature of the transmissible agent/s involved in the pathogenesis of TSE. The early occurrence of retinopathy in experimental spiroplasmosis in ruminants and in natural scrapie may serve as a template to develop a pre-clinical test for TSEs.

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**REFERENCES**


**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article.

**Figure S1.** N2a mouse neuroblastoma cell culture inoculated with scrapie sheep isolate.

**Figure S2.** Suckling mouse cataract (SMCA) infection of bovine corneal endothelia (BCE) cell culture.

**Figure S3.** Suckling mouse cataract (SMCA) and scrapie spiroplasma isolates showed multiformity in M1D media and bovine corneal endothelia (BCE) tissue culture fluid.

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