Immunohistochemical investigation of the damage to and repair of myelin, and astrocyte activity in small ruminants resulting from with natural meningoencephalitic listeriosis

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Summary: Meningoencephalitic listeriosis is a significant bacterial disease in various species. Common characteristics include microabcesses in the brainstem, midbrain and cerebellum. Many aspects of damage to the central nervous system caused by the disease remain obscure. We investigated central nervous system damage by assessing the presence of biomarkers such as galactocerebrosid (GAL-C), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) in brain tissues of sheep and goats of various ages, which naturally infected with listeriosis. We examined sections of pons, medulla oblongata, rostral colliculus, cerebellum, thalamus and cornu ammonis and found increased MBP (P<0.01), GAL-C (P<0.01) and GFAP (P<0.01) expression. This study showed that myelin damage in meningoencephalitic listeriosis was an important neuropathological finding. The most prominent finding of this study is the beginning of the regeneration as soon as myelin damage occurs.

Keywords: Astrocytes, GAL-C, GFAP, Listeria monocytogenes, MBP.

Introduction

Listeriosis is a zoonotic bacterial infection caused by Listeria monocytogenes. The disease is characterized by abortion, mastitis, meningoencephalitis and septicemia. Except for the meningoencephalitic form, clinical findings are nonspecific (2, 21). Although microabcesses, glial nodules and perivascular mononuclear cell infiltration in the brainstem are characteristic of listeriosis, a definitive diagnosis can be made by identification of the infective agent using immunopathology and electron microscopy or by isolating bacteria from the tissue (13, 21). Use of an immunoperoxidase staining technique has been reported to enable rapid and sensitive diagnosis of both cerebral infection and the abortive septicemic and subclinical forms of the disease (12, 13, 21).

Astrocyte and oligodendrocyte activity are observed during central nervous system repair. This repair involves an increased number of astrocytes and neighboring destroyed neurons, which is also known as astrocytosis. During astrocytosis, marked increase is observed in astrocytes that have star-shaped extensions and swollen cytoplasm. Damaged areas are surrounded by astrocytes, which form a type of scar tissue. During the repairment period, immunohistochemical staining of glial fibrillar acidic protein (GFAP) in the astrocytes is strong (9, 19). Oligodendrocytes adjacent to healthy neurons are believed...
to connect these neurons to blood vessels. Oligodendrocytes also are responsible for the characteristic appearance of satellitosis as these cells wrap themselves around degenerated neurons and participate in the repair of damaged myelin sheaths. When oligodendrocytes become active, fibrillar structures develop. And galactocerebroide (GAL-C) can be detected by immunostaining (19). Destruction of myelin sheaths is indicated by the presence of myelin basic protein (MBP), which is detectable by immunohistochemical staining (9, 19).

We investigated central nervous system of small ruminants which naturally infected by L. monocytogenes and exhibited neurological signs. Bacteria were detected using anti-L. monocytogenes specific antibody visualized using a streptavidin-biotin peroxidase method. In addition, astrocytes, oligodendroglia and myelin antigens were visualized using GFAP, GAL-C and MBP primary antibodies, respectively.

The aim of this study is to investigate the relationship between destruction of myelin sheaths and neurological signs in animals naturally infected by Listeria monocytogenes and to demonstrate whether there is a correlation between neuropathology seen in meningoencephalitic listeriosis and damaged myelin sheaths repairment period. The activity of the astrocytes were also assessed in terms of its GFAP expressions in infected animals and healthy control groups.

Materials and Methods

Study material: Paraffin blocks of brain tissue diagnosed with meningoencephalitis from 11 sheep (2 months to 3 years old) and one goat (1-year-old), obtained from the archive of the Department of Pathology, Faculty of Veterinary Medicine, University of Kırıkkale were used. These samples had been collected between 2003 and 2009. Five samples with no brain lesions constituted the control group. Tissues from the pons, medulla oblongata, rostral colliculus, cerebellum, thalamus and cornu ammonis were selected for study. Also, one slide from a case diagnosed as canine distemper was used as a positive control, which was characterized by severe demyelination of the cerebellum. Listeria cases were numbered from L1 to L12 according to the date they were obtained; details are given in Table 1.

Histopathology: Sections 5 µm thick were cut from the blocks and all slides were stained with both hematoxylin and eosin (H&E) and Kluver-Barrera (luxol fast blue) methods (15) and mounted with cover slips. Sections of tissue blocks were evaluated in three groups: brainstem (pons, medulla oblongata, rostral colliculus), midbrain (thalamus, cornu ammonis,) and cerebellum. Three randomly selected fields were assessed for each slide using a light microscope at 20X. Gliosis, microabscesses, neuronal degeneration, perivascular mononuclear cell infiltrates and meningitis were assessed in each section and assigned a numerical score. The scoring system is described in Table 2 and the results are summarized in Table 3.

Table 1. Numbers of animals with neural symptoms suspected to be due to meningoencephalitic listeriosis. Data were collected 2003–2009.

| Case no. | Year | Species and age | Clinical signs | Province of origin |
|----------|------|----------------|---------------|-------------------|
| Control 1 | 2009 | Lamb, 1 year | No            | Kırıkkale         |
| Control 2 | 2009 | Goat, 8 months | No            | Kırıkkale         |
| Control 3 | 2009 | Lamb, 6 months | No            | Ankara            |
| Control 4 | 2009 | Goat, 1 year  | No            | Ankara            |
| Control 5 | 2009 | Lamb, 3 months | No            | Kırıkkale         |
| L1       | 2003 | Lamb, 3 months | Tremor excitation | Kastamonu         |
| L2       | 2003 | Lamb, 5 months | Tremor excitation | Kastamonu         |
| L3       | 2004 | Sheep, 3 years | Death after neural signs | Balkesir         |
| L4       | 2004 | Sheep, 2 years |               | Bolu              |
| L5       | 2004 | Lamb, 4 months | Torticollis circling | Ankara          |
| L6       | 2004 | Lamb, 5 months | Torticollis circling | Ankara          |
| L7       | 2004 | Lamb, 2 months | -             | Kırıkkale         |
| L8       | 2004 | Goat, 1 year  | -             | Kırıkkale         |
| L9       | 2005 | Sheep          | -             | Kırıkkale         |
| L10      | 2005 | Lamb           | -             | Kırıkkale         |
| L11      | 2006 | Sheep, 3 years | Animal was dead | Kırşehir          |
| L12      | 2009 | Lamb           | Death after birth | Kırıkkale        |
Table 2. Scoring criteria.
Tablo 2. Skorlama kriterleri.

| Score | Microabcess | Gliosis | ND and N | PVMI | MNG |
|-------|-------------|---------|----------|------|-----|
| 4     | ≥ 3 foci included 20 and more neutrophils | ≥ 3 foci | ≥ 10 necrotic neurons | ≥ 5 foci | More severe |
| 3     | 2 foci included 20 ≥ neutrophils | 2 foci | 6–9 necrotic neurons | 3–4 foci | Severe |
| 2     | 1 focus included 20 ≥ neutrophils | 1 focus | 1–5 necrotic neurons | 2 foci | Mid |
| 1     | 1 foci included 10–20 neutrophils | Mild glial proliferation | Neuronal shrinkage and chromatolysis | 1 focus | Mild |
| 0     | No lesions | No lesions | No lesions | No lesions | No lesions |

ND: neuronal degeneration; N: necrosis; PVMI: perivascular mononuclear cell infiltration; MNG: meningitis.
ND: nöronal dejenerasyon; N: nekrozis; PVMI: perivasküler mononükleer hücre infiltrasyonu; MNG: meningitis.

Table 3. Histopathological lesion scores.
Tablo 3. Histopatolojik lezyon skorları.

| Case no. | Microabcess | Gliosis | Neuronal degeneration | Perivascular cell infiltration | Meningitis | TLS |
|----------|-------------|---------|-----------------------|-------------------------------|------------|-----|
|          | BS | MB | C | BS | MB | C | BS | MB | C | BS | MB | C |
| L1       | 4  | 4  | 2  | 4  | 4  | 1  | 4  | 4  | 2  | 3  | 3  | 3  |
| L2       | 4  | 3  | 2  | 4  | 3  | 2  | 3  | 4  | 3  | 3  | 3  | 3  |
| L3       | 3  | 2  | 0  | 1  | 2  | 2  | 1  | 2  | 1  | 1  | 1  | 4  |
| L4       | 4  | 0  | 1  | 3  | 2  | 1  | 4  | 3  | 2  | 4  | 4  | 2  |
| L5       | 3  | 2  | 2  | 3  | 2  | 1  | 4  | 2  | 2  | 3  | 3  | 1  |
| L6       | 1  | 2  | 3  | 3  | 3  | 4  | 1  | 4  | 4  | 3  | 1  | 1  |
| L7       | 3  | 4  | 0  | 4  | 4  | 1  | 4  | 4  | 2  | 4  | 4  | 2  |
| L8       | 2  | 3  | 3  | 4  | 2  | 1  | 4  | 2  | 1  | 4  | 3  | 2  |
| L9       | 2  | 1  | 3  | 3  | 4  | 2  | 4  | 4  | 1  | 2  | 1  | 3  |
| L10      | 0  | 0  | 1  | 2  | 0  | 2  | 2  | 1  | 3  | 1  | 1  | 4  |
| L11      | 0  | 1  | 1  | 2  | 3  | 2  | 2  | 3  | 1  | 1  | 4  | 0  |
| L12      | 1  | 1  | 0  | 2  | 2  | 1  | 2  | 2  | 1  | 2  | 2  | 0  |
| Total    | 21 | 18 | 10 | 26 | 22 | 13 | 26 | 25 | 16 | 24 | 26 | 15 |

BS: brainstem; MB: midbrain; C: cerebellum; TLS: total lesion score H&E stained sections.
BS: beyin sapı; MB: ortabeyin; C:beyincik; TLS: H&E boyalı kesitlerde toplam lezyon skoru.

Table 4. Immunoperoxidase test results and statistical data.
Tablo 4. Immünoperoksidaz test sonuçları ve istatistiksel veriler.

|          | N  | Mean | Std. Deviation | Std. Error | P     |
|----------|----|------|----------------|------------|-------|
| GFAP     |    |      |                |            |       |
| Control animals | 5  | 2.938| 0.219          | 0.098      | 0.003 |
| Infected animals | 12 | 4.987| 0.410          | 0.137      |       |
| GAL-C    |    |      |                |            |       |
| Control animals | 5  | 2.072| 0.132          | 0.059      | 0.003 |
| Infected animals | 12 | 3.391| 0.416          | 0.139      |       |
| MBP      |    |      |                |            |       |
| Control animals | 5  | 1.344| 0.186          | 0.083      | 0.003 |
| Infected animals | 12 | 3.013| 0.338          | 0.113      |       |
Histopathology: Sections 5 µm thick were cut from the blocks and all slides were stained with both hematoxylin and eosin (H&E) and Kluver-Barrera (luxol fast blue) methods (15) and mounted with cover slips. Sections of tissue blocks were evaluated in three groups: brainstem (pons, medulla oblongata, rostral colliculus), midbrain (thalamus, coru ammonis,) and cerebellum. Three randomly selected fields were assessed for each slide using a light microscope at 20X. Gliosis, microabscesses, neuronal degeneration, perivascular mononuclear cell infiltrates and meningitis were assessed in each section and assigned a numerical score. The scoring system is described in Table 2 and the results are summarized in Table 3.

The total lesion score, which indicated the severity of the lesions, was obtained by adding the numerical scores along the rows from the left to right for each animal in Table 3. In this way, the most severe cases could be identified, which allowed us to relate the effects of factors such as age and species to prognosis of the disease. A score ≥ 30 was considered as severe while lower scores were considered as mild.

Affected brain areas with lesions were counted by adding the numerical scores in each column from up to down for each animal in Table 3. We determined that more severe brain lesion in which region. A score ≥ 20 was considered as severe; lower scores were considered as mild.

Immunohistochemistry:

Detection of Listeria: Immunohistochemistry was performed on sections of brain stem, midbrain and cerebellum to determine the distribution of *Listeria* specific antigens. All steps were carried out following the procedure described by Dinçel and Kul (5). Sections were incubated with rabbit-anti-*Listeria monocytogenes* sera diluted at 1:500 Antibody Diluent (S0809; DAKO, Denmark) for 60 min at room temperature. We used a commercial streptavidin-biotin complex peroxidase (ABC-P) kit according to the supplier’s protocol (LSAB2 Kits, Universal, K0675 HRP, Rabbit/Mouse, DAKO) for visualization of all immunohistochemical staining. 3-amino-9-ethylcarbazole was used as the chromogen with counterstaining by Mayer's hematoxylin. The same procedure was applied to the tissue sections of the control group animals.

Gliafibrillary acidic protein (GFAP), galactocerebrosid (GAL-C) and myelin basic protein (MBP) expressions: Serial tissue sections of experimental and control groups also were stained with the ABC-P method using anti-GFAP (1:100 dilution), anti-MBP (1:100 dilution) and anti-GAL-C (1:500 dilution) specific commercial antibodies. A case diagnosed as canine distemper, which is characterized by severe demyelination of the cerebellum, was used as a positive control for GFAP, MBP and GAL-C staining. Negative controls consisted of sections incubated with normal goat serum instead of the primary antibody prior to performing GFAP, MBP, GAL-C immunostaining.

The density of immunopositive staining was measured using a computerized image system composed of a Leica CCD camera DFC420 (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK), connected to a Leica DM4000 B microscope (Leica Microsystems Imaging Solutions, Ltd.) was used according to the procedure described by Dinçel and Kul 2015 (6). Five representative fields were selected under high-power view and consecutive pictures were captured by the Leica QWin Plus v3 software by a 20X objective (Leica Microsystems Imaging Solutions) at a setting identical to the image system for analyzing. For examining the staining of each antibody, we used the same setting for all slides. Integrated optical density of all positive stainings of GFAP, GAL-C and MBP in each photograph were measured. For the quantification of the mean GFAP-, GAL-C- and MBP- positive area/total area were measured and calculated by Leica Qwin Plus on the pictures. All images were collected under the same lighting conditions. Data were statistically described in terms of mean and standard deviation (mean±SD) for area %. After calculating the proportion (% pixels) of stained area to the whole field, the mean (in % pixels) staining area for each slide was determined. Statistical analysis of GFAP, GAL-C and MBP immunohistochemical results were compared between groups using Mann Whitney U test. The data were presented as mean±SD.

Results

Histopathological findings: The histopathology of the brainstem, midbrain and the meninges were investigated using the H&E stained sections. Typical multifocal microabscesses (Figures 1A, B) and perivascular mononuclear cell infiltration were detected near the brainstem. In addition, meninges covering the brainstem exhibited acute inflammatory cell infiltration with a predominance of neutrophils (Figure 1B). Meninges were thickened and inflammatory cells were observed migrating from vessel walls to meninges in lesioned areas. Perivascular mononuclear cells (predominantly lymphocytes) and microabscesses were observed adjacent to themes aquaductus mesencephali. Microglial proliferation (gliosis) was evident around the microabscesses. Astrocyte proliferation, neuronal degeneration and satellitosis (Figure 1B) were detected in lesioned regions including brain stem, midbrain and cerebellum gray matter. Some cases (L5, L6, L9) exhibited no lesions around listeriosis-specific microabscess, no perivascular mononuclear cell infiltration and no signs of meningitis and these cases were
excluded from our study. Lesion scores are summarized in Table 3. The brainstem, cerebellum and midbrain were the parts of the brain affected most adversely. Cases L1–L8 were considered severe and the remainder were considered mild based on criteria described in the Methods section. Age or species predisposition were not detected among cases (Table 3). We observed that the most affected area was the brain stem (9 cases).

**Luxol fast blue findings (LFB):** Listeria monocytogenes-infected animals showed significantly blanched areas of demyelination, while high intensity staining was evident in the healthy-control group. LFB staining revealed significant demyelination in the white matter of the cerebellum and brainstem in the Listeria monocytogenes-infected animals (Figure 1D).

**Immunohistochemical findings:** Listeria spp. were confirmed by streptavidin-biotin peroxidase methods in selected tissue sections. We observed immunopositive Listeria spp. in microabscesses and perivascular mononuclear cell infiltrations in brain parenchyma and meninges (Figure 1C). We examined reactive astrocytes (gemistocytic astrocytes) in both gray and white matter, and these showed increased anti-GFAP immunopositive areas compared to control group ($P<0.01, 4.987-0.41$) (Figure 2A). The control group tissue sections exhibited fewer stained organisms than the listeriosis group (Figure 2B). We also observed intensely positive MBP staining as disorganized linear or particulate entities, especially in damaged areas ($P<0.01, 3.013-0.338$) (Figure 2C). In control group sections, stained material appeared as regular cords or rows (Figure 2D). Strong GAL-C staining was observed in oligodendrocyte cytoplasm surrounding degenerated neurons, demyelinated areas and perivascular regions ($P<0.01, 3.391-0.416$) (Figure 2E). By contrast, weak GAL-C staining was observed in the control group sections (Figure 2F).

Statistical analysis of the data on MBP, GAL-C and GFAP expression in the brain, measured by immunostaining in all the groups, are listed in Table 4.
Figure 2. A) Sheep, case no. L2. Intense GFAP staining of the pons showing numerous stellate cells (arrow). Streptavidin-biotin peroxidase method, Bar=100μm. B) Sheep, control no 1. Weak GFAP staining of the pons in a control group animal (arrow). Streptavidin-biotin peroxidase method, Bar=100μm. C) Sheep, case no. L7. Disorganized MBP stained areas among inflammatory cell infiltration demonstrating myelin cluster residues in the rostral colliculus (arrow). Streptavidin-biotin peroxidase method, Bar=100μm. D) Sheep, control no 3. MBP stained cords or rows in the rostral colliculus of a control animal (arrow). Streptavidin-biotin peroxidase method, Bar=50μm. E) Sheep, case no. L10. Strong GAL-C staining of oligodendrocytes (arrows) in the thalamus. Streptavidin-biotin peroxidase method, Bar=50μm. F) Sheep, control no 5. Oligodendrocyte (arrow) from the thalamus of a control animal unstained for GAL-C. Streptavidin peroxidase method, Bar=50μm.
Discussion and Conclusion

Listeriosis is a common zoonotic bacterial disease in domestic cattle, goats and sheep (1). Adult goats and sheep are particularly sensitive to this disease (3), but outbreaks also have been reported in lambs (16, 20). Previously, the axonal degenerations caused by Listeria monocytogenes are described (10). However, there is an in vitro study in which MBP expressions are examined (18). However, limited studies have investigated the particular mechanisms of myelin degeneration and regeneration in meningoencephalitic listeriosis (11). In this study, it was demonstrated that in meningoencephalitic listeriosis observable regenerations were detected as soon as myelin degeneration occurred. In addition to these findings, it has been shown that myelin damage in meningoencephalitic listeriosis was an important part of neuropathological evaluations.

Listeria is a widely distributed flagellated bacterium that is resistant to many environmental conditions. In addition, Listeria can be spread by feces, nasal and vaginal discharges, and tears (9). Consequently, Listeria is observed most commonly in intensive animal farming. Compero et al. (2002) (3) reported that morbidity is greater in sheep than cattle owing to poorer housing and feeding conditions, and greater stress. Small ruminants in Turkey commonly are raised and fed in fields with negligible use of silage. Accordingly, the animals investigated in this study were also fed with a similar diet. We suspect that infection may have occurred due to contaminated feed or water.

How Listeria reaches brain tissue remains a matter of debate. Some claim that this occurs through the trigeminal nerve from the nasal or oral mucosa or from the conjunctiva (1, 3, 7). Others suggest that Listeria reach the central nervous system as a result of septicemia. Listeria can cross the placental barrier, which is somewhat similar to the blood-brain barrier (4, 14). Our total lesion score data indicate that the most severe lesions were in the brainstem and midbrain. Neuronal degeneration and gliosis in those regions suggest that Listeria migrate through neurons and that neuronal degeneration evokes inflammation. Therefore, inflammatory infiltrates in the form of perivascular mononuclear cell infiltration are located in the brainstem and midbrain following neuronal degeneration.

Nevertheless, it is still unclear how Listeria reaches the meninges and it requires further investigation. Ondorff et al. (17) reported that Listeria reached the meninges owing to septicemia, but did not explain how the organisms crossed the blood-brain barrier. Dramsi et al. (8) reported that Listeria can survive in phagocytic cells and can spread to the meninges from brain parenchyma through the cerebrospinal fluid, blood and lymph circulations. Concomitant to the previous study we observed Listeria immunostaining within phagocytic cells in the meninges.

Astrocytes participate in repairing damaged parts of the brain. The presence of reactive astrocytes can be detected by GFAP expression in brain tissue (9, 19). We found that GFAP expression was increased more in severe cases than in mild cases of listeriosis in the experimental group. We demonstrated that the expression of the GFAP is directly related to the severity of the lesions of Listeria.

We also found that more MBP is present in Listeria infected animals than in healthy animals, which indicates that myelin damage plays an important role in listeriosis; myelin damage is also greater in more severe infections. The most striking finding of this study was that there was a positive correlation between GAL-C and MBP expressions. This positive correlation showed that together with degenerations of the myelin, regeneration was observed at the same time. However, according to the LFB findings, the regeneration of myelin from the spindle cannot be fully achieved. It is evident from these findings that the destructive effects of the agent are more intense than the regenerative process of metabolism. Therefore, a complete myelin regeneration should not be expected during the infectious period.

There are limited studies on demyelination in listeriosis (11). The pathogenesis of myelin damage is still not fully understood. In this study, the existence of myelin damage has been revealed. However, it is interesting that MBP expressions are increased. In addition, myelin damage was revealed with LFB. On the other hand, myelin did not disappear and presence of MBP expressions in microenvironment. This study shows that myelin damage from listeriosis is out of the ordinary situation. There is myelin damage in this disease but myelin is also present in the environment. The same increase in GAL-C expressions confirms this theory. The same intensity increase of GAL-C and MBP expressions that are in harmony with each other is a significant finding in listeriosis, in which myelin damage is observed. Demonstrating a significant increase in GAL-C and MBP expressions despite the presentation of myelin damage, suggests that pathogenesis of myelin damage associated with listeriosis is quite complicated.

In conclusion, it has been shown that myelin damage has a very important neuropathology that occurs in the meningoencephalitic listeriosis. This study also showed that oligodendrocytes did not lead the target glial cells of the agent. More studies are needed to investigate the pathogenesis of myelin damage in the field.

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