The pathology of peracute experimental *Clostridium perfringens* type D enterotoxemia in sheep

F. A. Uzal,¹ W. R. Kelly, W. E. Morris, J. Bermúdez, M. Baisón

Abstract. The pathological findings in sheep with peracute experimental *Clostridium perfringens* type D enterotoxemia are described. Of 16 animals inoculated intraduodenally with a whole culture of this microorganism and a starch solution in the abomasum, 12 developed clinical signs including increased respiratory efforts, recumbency, paddling, bleating, convulsions, blindness, and opisthotonus. Diarrhea was not observed in any of the animals. The time lapse between the beginning of intraduodenal infusion and onset of clinical signs varied between 30 minutes and 26 hours, and the clinical course varied between 1 and 9 hours. Gross postmortem changes were observed in these 12 animals and included pulmonary edema; excess pericardial, peritoneal, or pleural fluid with or without strands of fibrin; liquid small intestinal contents; leptomeningeal edema; cerebellar coning; and subcapsular petechiae on kidneys. Histological changes consisted of severe edema of pleura and interlobular septa and around blood vessels and airways and acidophilic, homogeneous, proteinaceous perivascular edema in the brain. Five of 12 animals (42%) with clinical signs consistent with enterotoxemia lacked specific histological lesions in the brain. None of the intoxicated or control animals developed nephrosis. Glucose was detected in the urine of 3 of 6 animals that were tested for this analyte. These results stress the importance of the use of histological examination of the brain, coupled with epsilon toxin detection, for a definitive diagnosis of *C. perfringens* type D enterotoxemia in sheep.

Introduction

The pathogenesis of *Clostridium perfringens* type D enterotoxemia in sheep has been partially elucidated by experimental studies on different animal species.¹⁻³,⁶⁻¹⁰,¹¹,¹³ Most of these models were based on intravenous inoculation of *C. perfringens* type D epsilon toxin in mice,⁶⁻¹⁰,¹¹ rats,¹² and sheep.⁸⁻¹³,¹⁴ Characterization of the pathology of enterotoxemia in sheep has therefore largely depended on animals inoculated intravenously with epsilon toxin.⁸⁻¹⁰,¹¹,¹³ However, the gross and microscopic pathology of animals inoculated intravenously with epsilon toxin does not always reflect the changes of the natural disease.

Descriptions of gross and microscopic changes in natural cases of *C. perfringens* type D enterotoxemia in sheep are scant.⁷,¹⁵ Also, few studies⁴⁻⁵,¹⁷ have involved the use of a model closer to the natural disease than the intravenous inoculation of epsilon toxin, i.e., inoculation of whole cultures of *C. perfringens* type D into the bowel, together with the introduction into the alimentary tract of a carbohydrate substrate to encourage the multiplication of this microorganism and production of toxins. However, the gross and microscopic pathology of enterotoxemia was not described in 2 of the mentioned studies,⁵,¹⁷ and only a brief reference to gross changes was made in the other study.³ Therefore, there are still misconceptions about the pathologic features of sheep enterotoxemia and the diagnosis of this condition. The uncertainties of diagnosis have been compounded by the flaws inherent in the microbiological and toxicological methods used for confirmation of the diagnosis.¹⁶,²⁰

An experimental model of enterotoxemia was used to precisely define what levels of antiepsilon toxin antibody are protective against enterotoxemia in sheep. This model was based on the experimental model used previously in goats¹⁹ and involved the use of a relatively large number of animals. The results of the antibody study will be published separately. The pathological findings in the animals with experimental enterotoxemia are presented here.

Materials and methods

Animals. Twenty Merino lambs, male and female, were used. They were born to mothers that had been regularly vaccinated against enterotoxemia and that had received the last vaccine booster approximately 1 mo before lambing. The lambs were weaned into pens at the age of 12–14 wk
and fed milk replacer, alfalfa hay, and water ad libitum for 1–2 wk before the experiments. All animals were between 12 and 14 kg body weight at the time of the challenge. The lambs were randomly allocated to 2 groups. Group 1 comprised 16 *C. perfringens*-treated animals, whereas group 2 comprised 4 control animals. Conventionally reared male Quackenbush mice (20–25 g body weight) were used for the mouse tests.

**Antiepsilon toxin antibody detection.** A blood sample was obtained from all lambs before inoculation. The sera were separated and frozen at −20 C until tested for antiepsilon toxin antibodies by a mouse neutralization test and an indirect enzyme-linked immunosorbent assay (ELISA) as previously described.1,21

**Inocula.** A freeze-dried *C. perfringens* type D strain (Strain NCTC 8346) was reconstituted in cooked meat medium (CMM) and incubated overnight at 37 C under anaerobic conditions. A 10% inoculum was seeded into 500-ml bottles of the same medium, which were further incubated anaerobically for 10 hr at 37 C. The liquid phase of these cultures was filtered through sterile gauze. The filtrate was mixed 1:9 with a 1% trypsin solution to activate epsilon protoxin and incubated at 37 C for 30 min. The trypsin-treated filtrate was used as inoculum (A) for group 1. Nontoxic, sterile supernatant of fresh CMM was trypsinized as described and used as inoculum (B) for group 2.

Before inoculation, samples of the inocula were obtained for the following determinations: 1) Samples of both inocula were inoculated on blood agar and incubated aerobically at 37 C for 48 hr to check for contamination. 2) Samples of inoculum (A) were used to determine the purity of the culture and colony-forming units (CFU) per milliliter in a standard spread technique on blood agar after anaerobic culture at 37 C for 24 hr. 3) Samples of inoculum (B) were inoculated into CMM and incubated anaerobically at 37 C for 24 hr to check for contamination. 4) An aliquot of inoculum (A) was centrifuged at 10,000 rpm for 25 min at 4 C and filtered through a 0.22-μm filter. This filtrate was activated with 0.1% trypsin and stored at 1% peptone water, pH 7.2, at 37 C for 40 min. The tryptic activity of this filtrate was used as inoculum (B) for group 2. Nontoxic, sterile supernatant of fresh CMM was trypsinized as described and used as inoculum (B) for group 2.

**Results**

No growth was observed in the aerobic cultures of any of the inocula or in the anaerobic culture of inoculum (B), whereas pure cultures of *C. perfringens* type D were obtained when the inoculum (A) was incubated anaerobically. Colony counts were from 2.4 × 10⁸ to 4.6 × 10⁸ CFU/ml. Epsilon toxin was identified in the supernatant of the inoculum (A) when tested by mouse bioassay. These specimens had concentrations of epsilon toxin of between 200 and 400 MLD₉₀/ml. Epsilon toxin antibody levels in the serum of the experimental animals collected immediately before the challenge were below 0.1 IU/ml in all cases.

All the animals recovered from the anesthetic within 1 hour after the end of the procedure. Twelve of 16 animals in group 1 developed clinical signs, necessitating euthanasia, within 26 hours of inoculation (Table 1). The interval between the commencement of intraduodenal infusion and onset of clinical signs varied between 30 minutes and 26 hours, whereas the interval between the commencement of clinical signs and euthanasia of the animals in this group varied between 1 and 9 hours (Table 1). Clinical signs in animals of group 1 included increased respiratory efforts, recumbency, paddling, bleating, convulsions, blindness, and opisthotonus. Diarrhea was not observed in any animal. No clinical signs were observed in the remaining 4 animals of group 1 or in any of the animals in control group 2.

Gross postmortem changes were observed only in the 12 animals of group 1 that had shown clinical dis-
Table 1. Time elapsed between dosing and onset of clinical signs and between onset of clinical signs and death, main clinical signs, and postmortem findings in 12 lambs dosed intraduodenally with *Clostridium perfringens* type D culture supernatants (group 1). Necropsy was performed immediately after death except for 3 animals.

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Between dosing and onset of clinical signs</th>
<th>Between onset of clinical signs and death</th>
<th>Main clinical signs</th>
<th>Main postmortem findings</th>
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<tbody>
<tr>
<td>1</td>
<td>30 min</td>
<td>3 hr</td>
<td>dyspnea</td>
<td>interstitial lung edema, leptomeningeal edema</td>
</tr>
<tr>
<td>2</td>
<td>2 hr</td>
<td>2 hr</td>
<td>dyspnea</td>
<td>excess pericardial fluid, watery jejunal content</td>
</tr>
<tr>
<td>3</td>
<td>3 hr</td>
<td>1 hr</td>
<td>depression, head tremors</td>
<td>excess abdominal fluid, lung edema</td>
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<tr>
<td>4</td>
<td>3 hr 30 min</td>
<td>3 hr 30 min</td>
<td>convulsions</td>
<td>excess pericardial fluid, watery jejunal content, leptomeningeal edema</td>
</tr>
<tr>
<td>5*</td>
<td>4 hr</td>
<td>2 hr</td>
<td>depression, head tremors, recumbency, paddling</td>
<td>excess pericardial and abdominal fluid, pericardial fibrin, watery colon contents</td>
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<td>6*</td>
<td>4 hr</td>
<td>6 hr</td>
<td>bleating, head tremors, recumbency, paddling, unconsciousness, dyspnea</td>
<td>excess pericardial fluid with fibrin strands, leptomeningeal edema</td>
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<tr>
<td>7</td>
<td>4 hr 15 min</td>
<td>5 hr</td>
<td>recumbency, convulsions</td>
<td>excess abdominal fluid, red papillae of kidneys</td>
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<td>8</td>
<td>4 hr 30 min</td>
<td>4 hr 45 min</td>
<td>recumbency, paddling, convulsions, frothing, blindness</td>
<td>excess pericardial fluid, watery duodenal content, pericardial petechiae</td>
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<td>9</td>
<td>5 hr</td>
<td>1 hr</td>
<td>depression, head tremors</td>
<td>excess abdominal and pericardial fluid, pericardial fibrin, lung edema</td>
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<td>10*</td>
<td>8 hr</td>
<td>2 hr</td>
<td>incoordination, head tremors, recumbency, opisthotonous, dyspnea</td>
<td>excess abdominal and pericardial fluid, watery small and large intestinal contents</td>
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<tr>
<td>11</td>
<td>8 hr 30 min</td>
<td>9 hr</td>
<td>convulsions</td>
<td>excess pericardial, thoracic, and abdominal fluid (clotted), cerebellar coning</td>
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<tr>
<td>12</td>
<td>26 hr</td>
<td>3 hr</td>
<td>incoordination, head tremors, recumbency, opisthotonous, dyspnea</td>
<td>subcapsular petechiae on kidney</td>
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* Animals in which necropsy was performed 6 hr after death.

ease. One or more of the following changes were observed in all those animals: variable amounts of froth in the trachea and bronchi; interlobular septa of the lungs widened by fluid; red, wet, heavy lungs (Fig. 1); excess pericardial fluid (Fig. 2) with or without strands of fibrin; excess abdominal or thoracic fluid; liquid small intestine content; and subcapsular petechiae on kidneys (Table 1).

In the animals that had gross postmortem changes in the lungs, there were histologic changes consisting of severe edema of pleura and interlobular connective tissue and around blood vessels and airways. This edema was slightly to moderately eosinophilic. A moderate amount of eosinophilic fluid was observed in the alveolar lumens. In the brain, acidophilic, homogenous, proteinaceous edema was observed surrounding blood vessels (Fig. 3) in the internal capsule, thalamus, cerebellar peduncles, or cerebellum (or all) of 7 lambs.
(Table 2; Fig. 4). Moderate perivascular hemorrhage was occasionally observed in these areas. These lesions were bilateral and symmetrically distributed, and both veins and arteries were approximately equally affected. No histological changes were observed in the kidneys of the lambs that were necropsied immediately after death (Fig. 5a), except for 1 lamb that showed multifocal renal cortical interstitial hemorrhage. The

Figure 2. Excess pericardial fluid and fibrin in the pericardial sac of a lamb given *Clostridium perfringens* type D whole culture intraduodenally. Reprinted from Anaerobe, Copyright 2003, with permission from Elsevier.

Figure 3. Proteinaceous fluid surrounding a blood vessel in the cerebellum of a lamb infused intraduodenally with *Clostridium perfringens* type D whole culture. HE. Bar, 60 µm.
Table 2. Histopathological changes in brains of 12 lambs dosed intraduodenally with Clostridium perfringens type D culture supernatants (group 1).

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Parietal cortex</th>
<th>Internal capsule</th>
<th>Putamen</th>
<th>External capsule</th>
<th>Thalamus</th>
<th>Midbrain</th>
<th>Cerebellar peduncles</th>
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+: no lesions observed; +, ++, +++: severity of lesions observed (in ascending order of severity)

...kidneys of the 6 lambs from groups 1 and 2 that were necropsied 6 hours after death had changes in the proximal and distal tubular epithelium consisting of nuclear pyknosis, karyorrhexis, and karyolysis (Fig. 5b). These renal changes were identical in animals from groups 1 and 2. No histological changes except varying degrees of mucosal congestion and moderate numbers of gram-positive rods in the lumen were seen in the small intestine or colon of any of the animals with clinical enterotoxemia.

Large numbers of short gram-positive rods with blunt ends were observed in smears of duodenum, ileum, and colon of all animals in group 1 that developed clinical signs. Rich, almost pure cultures of C. perfringens type D were obtained from ileum of these animals, and epsilon toxin was detected in the ileal content of all the lambs of group 1 but not in the ileal contents of any of the control animals. A mixed population of gram-positive and gram-negative bacteria (including a few gram-positive rods) was observed in smears of small and large intestine of the animals that did not show clinical signs (groups 1 and 2). Clostridium perfringens type D was also isolated, although in small numbers (usually less than 2 colonies per plate) and in mixed cultures, from the ileum of the 4 animals of group 1 that did not develop clinical disease.

Glucose was detected (2,000 mg/dl) in the urine of 3 of the animals of group 1. No urinary glucose was detected in the other 3 animals of group 1 that were tested for this analyte or in any of the animals of group 2.

**Discussion**

The protocol used here provides a reproducible model of naturally occurring enterotoxemia in sheep, and the use of this experimental model has removed some of the variation that is inherent in studies using intravenous epsilon toxin or field cases of enterotoxemia, but there are still several variables to bear in mind while analyzing these results.

First, there was some variation in the interval between inoculation and onset of clinical signs (less than...
Figure 5. Kidneys of lambs infused intraduodenally with *Clostridium perfringens* type D whole culture and fixed immediately (a) or 6 hours (b) after death. HE. There are no significant lesions in (a), and (b) exhibits marked autolysis. Bar, 60 μm.

1–26 hours) and between onset of clinical signs and death (1–9 hours). Thus, there was presumably considerable variation in duration of action of epsilon toxin on the tissues of the experimental group, the result of which could have been variation in clinical signs, gross and microscopic pathological changes, and urinary glucose content. Second, most animals affected in this experiment were killed for humane reasons, instead of dying spontaneously, and this factor would have further shortened the duration of action of the
toxin on target tissues. Third, in most of these experimental cases, the interval between euthanasia and necropsy sampling was much shorter than is the case for most necropsies on field cases.

Some or all of these factors may have resulted in differences between the lesions of the experimental and natural disease. Table 1, however, shows that there is no real relationship between the interval between inoculation and onset of clinical signs on the one hand and the nature and severity of the gross necropsy findings on the other. There is, however, a distinct relationship between the interval between onset of clinical signs and euthanasia and the presence of microscopic lesions in the brain: i.e., the 4 animals with the shortest interval were among the 5 animals that did not develop perivascular proteinaceous edema (microangiopathy) of the brain (Table 2).

A difficulty faced when designing this protocol was to obtain an inoculum that reproduced as closely as possible the clinical signs and postmortem changes of natural enterotoxemia. Although it is difficult to establish a priori the volume and concentration of such an inoculum, the clinical and pathological results of the experiments in this study suggest that the inoculum used in this study reproduced a condition similar to the natural disease.

Twelve of the 16 animals in group 1 developed disease, whereas the other 4 animals did not develop clinical disease or lesions. In previous studies on experimental sheep enterotoxemia induced by inoculation of *Clostridium perfringens* in the bowel, only few animals were used and although most sheep developed disease, the number of animals was too small to draw definitive conclusions. However, all the mentioned studies used very large inocula, and this could have been responsible for the higher successful rate in reproducing sheep enterotoxemia. A similar model to the one described in this report was used previously in goats, and 13 of 17 inoculated goats developed clinical signs and lesions of enterotoxemia, a success rate similar to the one reported here. The reason why 4 of the 16 inoculated lambs in the present study did not develop clinical disease or lesions was not determined, but possible explanations include individual genetic variation to the disease and slight variations in the size of the inocula. A similar situation occurs in natural cases of the disease, when morbidity-mortality is never 100%.

In the present study, microangiopathy in brain sections was found in 7 of 12 animals dying of enterotoxemia. This change is characteristic of field enterotoxemia in sheep, and it is a valuable diagnostic criterion, particularly when intestinal content for epsilon toxin assay is not available. However, in this study, 5 of 12 lambs dying of enterotoxemia did not have brain changes. Four of these lambs were among those with the shortest dosing–euthanasia interval (Table 2). These were experimental cases that were euthanized for humane reasons, and it is possible that at least some of these animals would have developed microangiopathy had they lived longer. It is not known how many field cases fail to develop microangiopathy, although cases of enterotoxemia in which microangiopathy of the brain is not seen are occasionally diagnosed (Uzal et al., unpublished observation). Diagnosticians should be aware that while cerebral microangiopathy is a useful indicator of enterotoxemia, absence of this lesion does not rule out a diagnosis of this disease in sheep.

Previous studies suggested that brain lesions associated with epsilon toxin are due to the action of this toxin on endothelial cells, which generates perivascular edema followed by hypoxia and necrosis of the perivascular tissues. However, a recent study showed that epsilon toxin can also leak into the brain and act directly on rat neurons, producing degeneration and necrosis of these cells in cases of subacute or chronic epsilon toxin intoxication. Although histological changes were not observed in neurons in this study, it is possible that they were directly affected by epsilon toxin and were responsible for the central nervous system (CNS) signs observed; this could explain why some of the animals with CNS signs did not have detectable microangiopathy.

The location of the brain lesions in the present study was similar, although not identical, to those described in previous studies, and this suggests that sampling should include the following locations: internal capsule, thalamus, midbrain, cerebellum, and cerebellar peduncles. The reason for the distribution of lesions in the brain is not known and requires further investigation.

No significant histological changes were found in the small or large intestine of any of the animals dying from enterotoxemia. This finding agrees with previous observations indicating that no major intestinal changes occur in sheep enterotoxemia. It has been demonstrated experimentally that when epsilon toxin is inoculated into ligated intestinal loops of sheep, no morphological alterations are produced in the small intestine, whereas severe inflammatory changes are produced in the colon. It has been suggested that in cases of enterotoxemia in sheep, epsilon toxin is readily absorbed in the small intestine thus not reaching the colon in concentrations high enough to produce lesions.

*Clostridium perfringens* type D enterotoxemia has been called historically “pulpy kidney disease,” and the changes in the kidneys of sheep dying from this disease have been called nephrosis. However, in the present study, with the exception of only 1 animal...
from group 1 that showed renal cortical hemorrhages, neither gross nor histological changes were observed in the kidneys of the lambs that were necropsied immediately after death, which supports the hypothesis that the so-called pulpy kidney lesion is a postmortem phenomenon. The assertion that pulpy kidney disease is a postmortem phenomenon suggests that epsilon toxin accelerates the postmortem decomposition of kidneys. Although this is possible, it has not been proven and no information is available in the literature comparing the speed of autolysis in animals with enterotoxemia and animals dying from other causes. But even if this was proven to be true (i.e., that autolysis is accelerated in animals dying from enterotoxemia) the evaluation of the speed of autolysis is a very subjective parameter of little, if any at all, diagnostic value. Histology of the kidney should, therefore, not be considered as a diagnostic indicator in sheep enterotoxemia.

Severe glycosuria was found in 3 of 6 lambs from group 1 in which urine glucose was measured. Glycosuria is considered an indicator of enterotoxemia in sheep. These results confirm that glycosuria may be associated with enterotoxemia. However, the converse does not apply, i.e., absence of glycosuria does not rule out a diagnosis of enterotoxemia in sheep.

In this study, excess pericardial fluid with or without fibrin strands was found in 8 of 12 animals dying of enterotoxemia. This change, when present, would support a presumptive diagnosis of enterotoxemia in sheep. In this study, glucose was not measured in pericardial fluid or other bodily fluids other than urine.

In this study, epsilon toxin was detected in the intestinal contents of the animals inoculated with *Clostridium perfringens* type D but not in the intestinal contents of the control animals. The amount of epsilon toxin in the intestinal content necessary to produce disease in sheep (diagnostic threshold) has not been determined, and it has been suggested that small amounts of this toxin can be found in the intestinal contents of normal animals without pathological effects. However, the results of this and a previous study suggest that, at least with the method of toxin detection used in this study, epsilon toxin is not detected in the small intestinal contents, unless it is present above the threshold to produce disease.

This study stresses the utility of histological examination of the brain, coupled with epsilon toxin detection in the intestinal contents to achieve a definitive diagnosis of enterotoxemia in sheep. Other postmortem changes, such as pulmonary edema and excess pericardial fluid, with or without fibrin, are relatively consistent changes that should help in establishing a presumptive diagnosis of this disease. However, it should be stressed that these changes are nonspecific, and the diagnosis of this disease should never be based on these changes alone. This study also shows that kidney lesions are not a characteristic of sheep enterotoxemia, and diagnosticians should not rely on kidney changes to establish a diagnosis of this disease. Intestinal changes do not seem to be characteristic gross or microscopic findings in enterotoxemia of sheep.

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**Sources and manufacturers**

a. Trypsin 1:250, Difco, Detroit, MI.
b. Promex 2, Apex Laboratories Pty Ltd., Somersby New South Wales, Australia.
c. Rupun, Bayer, Buenos Aires, Argentina.
e. Xylocaine, Astra, Buenos Aires, Argentina.
g. Cobum 9, Bayer, Buenos Aires, Argentina.

**References**