Use of Bacitracin in the Prevention and Treatment of Experimentally-induced Idiopathic Colitis in Horses

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ABSTRACT

Ten healthy ponies from a single herd were found by repeated fecal culture to be free of *Salmonella* species and *Clostridium cadaveris*. In a preliminary study, four ponies administered a single oral dose of 10 mg/kg lincomycin did not develop idiopathic colitis when the drug was administered alone. Four other ponies were administered 10 mg/kg lincomycin by stomach tube together with 0.45 L of colonic content from a horse with idiopathic colitis induced earlier by lincomycin alone. Two of the four ponies were treated with 25 g oral zinc bacitracin premix (110 g/kg active ingredient) 24 h later. Forty-two hours after inoculation the two untreated ponies had severe signs of idiopathic colitis and were euthanized. Postmortem findings were typical of idiopathic colitis. The two treated ponies had milder illness but the more severely affected was also euthanized; the other was retreated at 42 h with bacitracin premix and again 12 h later. Its illness and diarrhea resolved over the next 24 h. *Clostridium cadaveris* was isolated in large numbers from the cecum of the euthanized ponies and their cecal content contained mouse lethal and guinea pig dermonecrotic, but not cytotoxic, activity. Enterotoxins of *Clostridium perfringens* and *Clostridium difficile* could not be demonstrated. No toxin could be demonstrated in culture supernatants of *C. cadaveris* or in supernatants of cecal contents treated with ethanol prior to culturing in anaerobically incubated broth. No *Salmonella* spp. were isolated. A further two ponies were administered 10 mg/kg lincomycin orally with 0.45 L colonic content from a horse with idiopathic colitis, as described. Twenty-four hours after inoculation these ponies were administered 50 g bacitracin orally on three occasions at 12 h intervals. Neither became ill with colitis.

The demonstrated value of bacitracin in the treatment and prevention of induced idiopathic colitis supports the pathogenetic role of a *Clostridium* species. We suggest that the *Clostridium* involved is a usually nonsporulating organism.

RÉSUMÉ

Pour cette étude, on a utilisé dix poneys d'un même troupeau dont les cultures bactériennes fécales étaient négatives pour la présence de *Salmonella* sp. et de *Clostridium cadaveris*. Lors d'une étude préliminaire, quatre poneys ayant reçu une dose orale unique de 10 mg/kg de lincomycine n'ont pas développé de signes de colite idiopathique. Chez quatre autres poneys, on a administré par voie nasogastrique, 10 mg/kg de lincomycine, de même que 0,45 L de contenu du côlon provenant de chevaux chez qui on avait précédemment induit une colite idiopathique. Deux des quatre poneys ont été traités, 24 heures plus tard, avec 25 g d'un pré-mélange de bacitracine de zinc (ingrédient actif, 110 g/kg). Quarante-deux heures après l'inoculation, les deux poneys non-traités ont démontré des signes sévères de colite idiopathique et ont été euthanasiés. Les changements post-mortem observés étaient caractéristiques des colites idiopathiques. Les deux poneys traités avec la bacitracine ont démontré des signes cliniques moins sévères, mais l'un d'eux a aussi dû être euthanasié. L'autre poney a été traité de nouveau avec le pré-mélange de bacitracine à 42 h et à 54 h. Les signes cliniques et la diarrhée ont disparu dans les 24 heures qui ont suivi. *Clostridium cadaveris* a été isolé en grand nombre du caecum des poneys euthanasiés et leur contenu cécal démontrait une activité léthale pour les souris et une activité dermonécrotique chez le cochon d'Inde, mais ne démontrait pas d'activité cytotoxique. La présence des entérotoxines de *Clostridium perfringens* et de *Clostridium difficile* n'a pu être démontrée. De plus, aucune toxine n'a été démontrée dans le surnageant des cultures de *C. cadaveris* ou dans le surnageant de la culture anaérobique du contenu cécal préalablement traité avec de l'éthanol. On n'isola pas de *Salmonella* spp. Chez deux poneys, on a administré 10 mg/kg de lincomycine oralement avec 0,45 L de contenu du côlon provenant d'un cheval atteint de colite idiopathique. Vingt-quatre heures après l'inoculation de ces poneys, on a administré à trois reprises, espacées de douze heures d'intervalle, 50 g de bacitracine oralement. Ces deux poneys n'ont pas démontré de signes de colite.

L'efficacité de la bacitracine pour le traitement et la prévention des colites idiopathiques induites expérimentalement suggère le pouvoir pathogène de *Clostridium* sp. Nous suggérons que les *Clostridium* affectés sont habituellement des organismes non-sporulants. (*Traduit par Dr Jean-Pierre Lavoie*)

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INTRODUCTION

Severe idiopathic colitis ("colitis X") is a well recognized sporadically-occurring acute, severe, and often fatal disease of horses but the causes are unclear (1-4). A previously reported method for inducing fatal idiopathic colitis in the horse involved oral administration of lincomycin to horses, followed by a small quantity of colonic content from horses dead with fatal idiopathic colitis (5,6). In resulting cases of severe colitis, *Clostridium cadaveris* was the predominant freely sporulating *Clostridium* species isolated from colonic ingesta (5,6). The study reported here was designed to support the likely clostridial basis of the experimentally-induced severe colitis by use of bacitracin in the treatment and prevention of the illness, as well as to identify the *Clostridium* spp. involved and to characterize the histopathological features of the disease.

MATERIALS AND METHODS

PONIES AND EXPERIMENTAL INFECTION

Ten healthy adult ponies were obtained from the university pony herd. Repeated fecal cultures did not demonstrate the presence of *Salmonella* species, *Yersinia enterocolitica* and *C. cadaveris* (7). The ponies were housed in pairs in isolation rooms. In experiment 1, group A (two ponies) and group B (two ponies) received 10 mg/kg lincomycin (Lincocin®, Upjohn, Tuco Products, Orangeville, Ontario) once by stomach tube, followed in group B by 50 g of oral zinc bacitracin premix (Zinc-Bacitracin®, Rhone-Poulenc Canada Inc., Mississauga, Ontario, active ingredient 110 g/kg) at 12 h intervals for three days. All ponies remained normal for the 12 days of this first study and they were reused for a second experiment. The four ponies again received 10 mg/kg lincomycin but also were given by stomach tube 3 L of *C. cadaveris* strain J1 (10^9 organisms/mL) cultured at 37°C for 36 h in thioglycollate broth (Difco, Detroit, Michigan), followed, in two ponies (group B), by 25 g zinc bacitracin premix at 12 h intervals for three days. In a third study, four ponies received 10 mg/kg lincomycin and 0.45 L of colonic content from a pony previously dead of colitis X (6). Oral zinc bacitracin premix (25 g) was administered to two of the four ponies for attempted prevention of the onset of colitis, and one was treated further with bacitracin. In a fourth study, a further two ponies were administered lincomycin and colonic content as described for the third study, but were treated 24 h later with bacitracin premix (50 g) on three occasions at 12 h intervals. All animals were monitored at regular, frequent intervals for development of diarrhea and clinical signs of colitis. As soon as clinical signs of moderate-severe colitis developed (heart rate >60, with colicky signs) affected animals were immediately euthanized and were necropsied without delay. Tissues were fixed in 10% formalin, embedded in paraffin blocks, sectioned and stained with hematoxylin-eosin according to standard methods. All experiments conducted on ponies followed the guidelines of the "Guide to the Care and Use of Experimental Animals" issued by the Canadian Council on Animal Care.

ISOLATION AND CHARACTERIZATION OF BACTERIA

To isolate readily sporulating clostridia, cecal content from untreated ponies with severe colitis in experiment 3 was diluted and washed in ethanol (8,9) and washed cecal dilutions spread onto prereduced anaerobic blood agar (Carr Scarborough Microbiologicals Inc., Stone Mountain, Georgia) and were inoculated into prereduced brain heart infusion broth (BHI) (10). The agar plates were incubated for five days anaerobically at 37°C and then examined and counted. Representative colonial types were subcultured on blood agar, then grown for 12 h in BHI. The culture supernatant filtrates were tested for toxicity in both the tissue culture, mouse lethality assay, and/or the guinea pig dermonecrotic assays. The most frequently isolated strains were identified to species by using their long chain fatty acid profiles and biochemical reactions (10). Cecal contents were tested for the presence of *Clostridium difficile* by a selective enrichment with cycloserine and cefoxitin (11). In addition, one gram samples of cecal contents were emulsified in 9 mL of buffered peptone and heated at 82°C for 10 min and then *Clostridium perfringens* isolation attempted using pour plates of tryptose-sulfite-cycloserine medium, incubated anaerobically, and selected black colonies confirmed as *C. perfringens* by determination of motility and biochemical tests (12). Attempts at *Salmonella* spp. isolation from ponies were by usual methods (7).

TOXIN ASSAYS

Mouse neutralization and guinea pig skin dermonecrotic assays were both done by the methods described in Sterne and Batty, as modified by Borriello and Carman (9,13). Cytotoxicity assays were done using cultured Chinese hamster ovary (CHO) cells (14). Cecal contents were tested for *C. difficile* toxin A (Tox A Test, TechLab Inc., Blacksburg, Virginia). *Clostridium perfringens* enterotoxin was assayed using a commercially available reverse passive latex agglutination kit (Oxoid Ltd., Basingstoke, England) and by a sandwich ELISA (15).

RESULTS

EXPERIMENTAL INFECTIONS

Ponies in the first study which received lincomycin orally showed no evidence of colitis for the 12 days of the study. The two ponies receiving bacitracin showed partial anorexia and became impacted and mildly icteric. Administration of bacitracin was stopped for this reason. The four ponies were then used for the second study, but showed no evidence of colitis following administration of lincomycin and *C. cadaveris*. The ponies receiving bacitracin were again mildly anorectic. In the third experiment, one pony developed early clinical signs indicative of colitis 24 h after oral administration of lincomycin and intestinal content. At this time, two ponies were treated with 25 g bacitracin premix orally. By 42 h postinoculation the two untreated foals had clinical signs of severe colitis and were euthanized. The two bacitracin treated ponies had milder illness but the more severe of the two was also euthanized because of elevated heart rate. The other was again treated with 50 g of bacitracin premix at this time and with 25 g 12 h

later. The diarrhea and colicky signs it was showing at 42 h postinoculation resolved over the next 24 h. When euthanized two days later it had well formed feces and no clinical evidence of colitis. In the fourth study, the two ponies administered lincomycin and colonic contents and, 24 h later, bacitracin premix (50 g) on three occasions at 12 h intervals, showed no clinical evidence of colitis. They were euthanized four days after the last bacitracin administration.

Gross morphological changes in the three affected ponies were limited to a mild increase in abdominal fluid present with minimal serosal discoloration of cecum and right ventral colon and, in the two untreated ponies, fibrin present on the serosal surface of cecum and large colon. Cecal and colonic content in all four ponies in experiment 3 was soft to liquid, and was most fluid in the two untreated ponies and in one treated pony which presented with clinical signs of typhlocolitis.

Histologically, the most prominent findings were also in the cecum and right ventral colon with varying degrees of involvement of left ventral, left dorsal, and right dorsal colon. In the two most severely affected ponies, the untreated ponies, there was attenuation or loss of the upper one third to one half of many crypts within cecum and large colon. Within the cecal mucosa of the three affected ponies there was dilation of crypts with neutrophils, exfoliated epithelial cells, and necrotic debris often present within affected crypts. Surface epithelium was often cuboidal or flattened or varying degrees of erosion of the surface epithelium was present. An overlying layer of exfoliated epithelial cells, neutrophils, lymphocytes, eosinophils, fibrin, erythrocytes and cellular debris often was adherent to the eroded mucosal surface. Mitosis in cryptal epithelium was common and goblet cells were reduced in number. There was occasional infiltration of crypts by neutrophils and eosinophils and central lymphocytolysis in several submucosal lymphoid follicles. Minimal to moderate numbers of mononuclear cells and eosinophils were present in submucosa; vessels throughout lamina propria and submucosa were moderately congested, a few small vessels within lamina propria and submucosa were thrombosed, and lymphatics were mildly dilated and rarely infiltrated by neutrophils.

ISOLATION AND CHARACTERIZATION OF BACTERIA

Cultures of feces, colonic contents and organs of ponies necropsied were all repeatedly negative for *Salmonella* spp. *Clostridium cadaveris* was detected in cecal contents from the three affected ponies in experiment 3 at numbers in excess of 10^6/g of wet content. The other clostridium present in similar numbers was identified as *C. paraputrificum*. Six other unidentified clostridia were detected at levels between 10^2 and 10^5 spores/g of content. Sporulating *C. perfringens* were detected in the affected, bacitracin treated, pony in experiment 3 at 10^6/g of cecal content but was absent (<10/g) from other ponies in this experiment. *Clostridium difficile* and *Clostridium spiroforme* were absent.

DETECTION OF CECAL CONTENT AND BACTERIAL CULTURE SUPERNATANT TOXICITY

Mouse lethal and guinea pig dermonecrotic activities were present in the cecal content of the two untreated ponies from experiment 3; cytotoxicity was not. Specific tests for the enterotoxins of *C. difficile* and *C. perfringens* were negative. When cecal content from each of the two untreated ponies in experiment 3 was diluted, treated with alcohol to remove nonsporeforming bacteria, and then cultured in BHI, no mouse lethal or guinea pig dermonecrotic toxin was detected in the culture supernatant. There was no cytotoxic activity in these preparations and *C. difficile* toxin was not detected. Using each of the three toxin assays described, no toxic activity was detected in BHI culture supernatants of isolates of *C. cadaveris*, *C. paraputrificum* or of the six unidentified clostridia recovered from each of the two untreated ponies in experiment 3. Culture supernatants from four other isolates of *C. cadaveris* from horses with severe experimentally-induced idiopathic colitis (6) were also negative in these assays.

DISCUSSION

In earlier studies of experimentally-induced severe idiopathic colitis, we found that oral administration of a single dose of lincomycin, with or without cecal content from horses with idiopathic colitis, consistently reproduced severe typhlocolitis in horses, usually within 60 h of administration (5,6). *Clostridium cadaveris* was consistently isolated in large numbers from the cecum of these horses but we were unable to reproduce the disease by oral administration of this organism (6). If lincomycin was given in addition to the *C. cadaveris*, the resulting colitis could not be distinguished from that when lincomycin was administered alone. In the present study, the unexpected finding that ponies in experiment 1 did not develop colitis when lincomycin was administered alone may be explained by the source of these animals, which was from an established pony herd maintained at pasture. It is possible that the bacterial agent of idiopathic colitis is not found either in the digestive tract of ponies and horses or the environment of the animals. By contrast, earlier studies have been conducted with ponies obtained from a stockyard and the animals, their environment, or both, were contaminated (5,6). By analogy, rabbits housed in plastic isolators do not succumb to antibiotic-induced *C. spiroforme* mediated diarrhea until challenged with the organism (16). In the second experiment, we were unable to reproduce colitis in ponies administered lincomycin and *C. cadaveris*, indicating that this organism is not the agent of idiopathic colitis in horses. This suggestion was confirmed by the inability to demonstrate toxic activity in culture supernatants of *C. cadaveris* isolated from ponies in experiment 3 or from earlier studies (6), as well as by the reports of others that *C. cadaveris* is nontoxigenic (17). The colitis induced in ponies in experiment 3, by oral administration of lincomycin and a small quantity of cecal content (containing the responsible agent) from a horse with experimentally-induced severe colitis, was typical on gross and microscopic pathological examination of idiopathic colitis (3,5,6).

The value of orally-administered zinc bacitracin in treatment (experi-

ment 3) or prevention (experiment 4) of experimentally-induced colitis was of exceptional interest for two reasons. Firstly, bacitracin is specifically active against gram-positive bacteria, thus supporting our hypothesis that a *Clostridium* species is the causative agent of idiopathic colitis. Secondly, this finding offers a simple, inexpensive and highly effective approach to treatment of idiopathic colitis in horses. Such an approach has the likely advantage of being selective only against gram-positive bacteria and thus of not deleting the majority protective gram-negative bacteria. While speculative, it is thus possible that inadvertent treatment of enteric salmonellosis with bacitracin would not have a deleterious effect on the course of the disease and might also prevent overgrowth of clostridial organisms. The dose of bacitracin premix to treat idiopathic colitis is somewhere between 25–50 g of premix on at least three occasions at 12 h intervals but is purely empirical at this stage. Since treatment with the higher dose was associated with reversible anorexia and large colon impaction, further studies are needed to establish the optimal dosing.

Cecal content from untreated horses with severe induced colitis had toxin(s) present as detected in all the assays used. This was not *C. difficile* type A toxin (18), tetanus toxin (19), or *C. perfringens* enterotoxin. No pathogenic, freely sporulating clostridia were detected in the cecal content of these ponies when sporeforming organisms were selectively isolated by alcohol treatment of content and enrichment with BHI broth. *Clostridium perfringens* enterotoxin could not be demonstrated and sporulating *C. perfringens* were absent from cecal content of two of the three ponies with colitis in experiment 3. This study does not support a role for *C. perfringens* enterotoxin in idiopathic colitis, suggesting that an earlier study reproducing hemorrhagic enterocolitis in ponies with intravenous injection of crude extracts of *C. perfringens* type A enterotoxin contained more than enterotoxin (20).

Our studies suggest that the agent of the experimentally-induced colitis we describe is a nonsporulating *Clostridium* species, which alcohol or heat treatment for isolation would have destroyed. A possible candidate is *C. perfringens*, which commonly fails to sporulate, and which produces a variety of toxins other than enterotoxin (17). This suggestion would be consistent with the work of Wierup who demonstrated a possible role for this organism in severe colitis in horses (21). Further studies will attempt demonstration of toxin from different toxin types of *C. perfringens* in the cecal content of ponies with experimentally-induced colitis and, if successful neutralization occurs, attempted culture using selective enrichment methods.

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