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Bismuth subsalicylate in the prevention of colonization of infant mice with *Campylobacter jejuni*

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SUMMARY

Infant mice were used for the evaluation of the efficacy of bismuth subsalicylate (BSS) in the prevention of the growth of *Campylobacter jejuni* in the intestine. The MIC_{90} of ten *C. jejuni* strains was 900 μg/ml. Of three dosage regimens tested, continuous treatment before and after the bacterial challenge, mimicking the way BSS is used in the prevention of traveller's diarrhoea, was the most effective. Growth inhibition was dose dependent; the high dose of 2000 μg per day was more effective than 300 μg per day. After cessation of treatment, campylobacter counts increased to the same level as in the control animals.

INTRODUCTION

Campylobacter jejuni is a common aetiological agent in traveller's diarrhoea. According to Finnish [1] and Swedish studies [2, 3], in approximately 70% of reported cases the illness was acquired during travel outside the Nordic countries. The severity of the clinical symptoms varied, but most cases recovered spontaneously and no antimicrobial treatment was needed. Prophylactic administration of some antimicrobial agents for the prevention of diarrhoea is rather common. In the 1970s, after the confirmation of the role of ETEC (enterotoxigenic *Escherichia coli*) as one of the major aetiological agents in traveller's diarrhoea, antimicrobial prophylaxis was directed more particularly to this organism [4]. One group of the drugs widely used for the prevention of diarrhoea is that of the different bismuth salts, including bismuth subnitrate, bismuth subgallate, and bismuth subsalicylate (BSS). In one study BSS reduced the occurrence of traveller's diarrhoea by 62% when 240 ml (17·5 mg/ml BSS) of the liquid formula was taken by mouth daily by US students in Mexico [56]. In another study, when the daily dose of bismuth subsalicylate was either 1·05 or 2·1 g on a twice-daily regimen, the diarrhoeal incidence was reduced by 35 to 41% [6]. In the studies mentioned above, campylobacters were not included in the microbiological diagnostic tests. The *in vitro* sensitivity of *C. jejuni* to bismuth compounds is not known.

In the present study, the efficacy of prophylactic or therapeutic dosages of bismuth subsalicyate in preventing the growth and colonization of *C. jejuni* in the intestinal tract of infant mice was investigated. The MIC value of bismuth subsalicylate to *C. jejuni* was also determined.

MATERIALS AND METHODS

Bacterial strains. The five *C. jejuni* strains used in the colonization studies were KH and 12650, isolated from patients with diarrhoea; B 6 and B 42, isolated from chickens; and L 69 isolated from a sheep. The strains were chosen because they were known to colonize the intestines of infant mice. These and 5 additional strains isolated from humans (2 strains), cattle (1 strain), and chicken (2 strains) were tested for *in vitro* sensitivity to bismuth subsalicylate. Before their use in the present study, the strains had been stored at −70 °C in Brucella FBP broth (Oxoid Ltd, UK).

Sensitivity testing. Bismuth subsalicylate (Orion Pharmaceuticals, Espoo, Finland) was first dissolved in 0·1 M-HCl. After 5 min at ambient temperature, the pH-value was adjusted to 7·0 with 1 M-NaOH. Serially diluted concentrations of BSS were then added to Brucella blood agar and Brucella broth with FPB supplement ($FeSO_4.7H_2O$, sodium pyruvate, sodium metabisulphite, each at concentration of 0·025 %). These were inoculated with 5 μl of a 24 h culture (37 °C) of campylobacters in Brucella FBP broth and the growth of bacteria was assessed after 48 h incubation at 37 °C.

Preparation of the challenge dose. The strains were grown in Brucella broth with 5 % sterile citrated calf blood at 37 °C for 24–30 h. The culture was diluted tenfold in 0·1 % peptone water and the number of colony forming units (c.f.u.) of the inoculum was determined by spreading 0·1 ml of appropriate dilutions on duplicate Brucella blood agar plates. The inoculum size used was 10^4–10^5 c.f.u. per animal. All incubations of broth or plate cultures were made microaerophilically.

Bismuth subsalicylate (BSS). BSS (Orion Pharmaceuticals, Espoo, Finland) is highly insoluble in water. Therefore a milky suspension of BSS in water was prepared each time for the treatment of animals.

Treatment of animals. The experimental animals used were 4 to 9-day-old infant mice. The adult females and males (NMRI; Orion Pharmaceuticals, Espoo, Finland) were mated and the infants were delivered to individually caged dams.

Three different treatment regimens were used: pre-treatment; post-treatment; continuous treatment ('prophylaxis'). The protocols are shown in Table 1. The bacteria and BSS were inoculated intragastrically, 0·1 ml each, using a 1·0 ml tuberculin syringe fitted with a blunted 21-gauge needle. The mice were killed by CO_2 asphyxiation. Control animals were inoculated with the bacteria and sampled in the same way but did not receive BSS treatment.

Testing of efficacy. The growth of *C. jejuni* was tested by counting the c.f.u.s of campylobacters either from the whole intestine or separately from the small and large intestine. The intestine was homogenized in 9·0 ml 0·1 % peptone water with a Colworth Stomacher Lab Blender (Tekmar; Cincinnati, OH). The homogenates were serially diluted in 0·1 % peptone water; and 0·1 ml of appropriate dilutions were spread on duplicate Blaser–Wang medium plates [7]. Similarly, the c.f.u.s of the challenge doses were counted on Brucella blood agar plates. After a 48 h incubation at 37 °C, the campylobacter colonies were counted.

Table 1. *Bismuth subsalicylate (BSS) treatment regimens in mice challenged with 10^4–10^5 c.f.u.* Campylobacter jejuni

	Day of experiment					
	1	2	3	4	5	6
Pre-challenge treatment	BSS 2000 μg daily		Challenge	Killed and tested		
Post-challenge treatment	Challenge	BSS 300 μg daily in two divided doses			Killed and tested	
Continuous treatment ('prophylaxis')	Challenge BSS 2000 μg or 300 μg daily in two divided doses					Killed and tested

RESULTS

Sensitivity of C. jejuni *to bismuth subsalicylate.* The sensitivity (MIC_{90}) of 10 *C. jejuni* strains, including the strains used in the mice experiments, as tested either by the broth or agar dilution methods, was 900 μg per ml (range 450–900 μg/ml).

Treatment experiments. None of the mice in any of the test or control groups showed signs of illness. The only visible sign, which was always seen in treated animals, was the dark greenish colour of the contents of the large intestine.

Pre-challenge treatment. The effects of 2 days of pre-treatment with BSS on the growth of *C. jejuni* in the intestine of infant mice are presented in Table 2. BSS administered to mice inhibited the growth of campylobacters. The difference of the means of c.f.u.s between control and treatment groups was significant ($P < 0{\cdot}001$). The sensitivity of the method was log 2 c.f.u per specimen. However, if the animals treated with BSS were killed 1 week after the bacterial inoculation, no difference in the counts (10^5–10^6) between the two groups was found indicating that the effect of prophylactic treatment is restricted to the treatment period.

Post-challenge treatment. Table 3 presents the effect of BSS used as a therapeutic agent. With the lower daily dose of 300 μg per animal for 3 days after the campylobacter challenge, the growth of the organism was not inhibited ($P < 0{\cdot}05$). Only one strain, KH, was tested using both low and high doses of BSS. The high dose was apparently effective in inhibition of the growth of *C. jejuni*. In the high dosage regimen, in 6 of the 12 animals tested, campylobacters were undetectable at the level of the sensitivity of the method. The results, however, are not directly comparable because of different challenge doses.

Continuous pre- and post-challenge treatment. Treating the animals for 2 days before and 3 days after the bacterial challenge caused a statistically significant prevention of the growth of *C. jejuni*. With the dose of 2000 μg per animal per day, the counts of campylobacters were under the detection level of log 2 in all (17) treated animals (Table 4). Further, the low dose of 300 μg per animal was also effective in prevention of the growth of *C. jejuni*. In the experiments with strains KH, B 42, and 9000, the counts of campylobacters were analysed separately for the small and large intestine. The growth of campylobacters was inhibited both in the small and large intestine. When, however, the counts of campylobacters were

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Table 2. *Effect of prophylactic treatment with bismuth subsalicylate* on the growth of* C. jejuni *in the intestine of infant mice*

Bacterial strain	Bacterial counts (log c.f.u) per intestine	
	Control animals	Treated animals
KH	5·18 ± 1·07 (5)†	2·41 ± 1·2 (4)
L69	4·75 ± 0·22 (8)	2·55 ± 0·57 (11)
12650	4·60 ± 0·60 (5)	2·93 ± 0·81 (14)
B42	4·5 ± 0·45 (4)	< 2·0‡ (4)

* Daily doses (2000 μg per animal) for 2 days before the bacterial challenge (10^4–10^5 c.f.u. per animal). The animals were killed 24 h after the challenge.
† Number of animals given in parentheses.
‡ Sensitivity of the detection method was log 2·0.

Table 3. *Therapeutic use of bismuth subsalicylate (BSS) for prevention of the growth of* C. jejuni *in the intestine of infant mice*

Bacterial strain/ challenge dose (c.f.u. per animal)	Daily dose of BSS per animal (μg)*	Bacterial counts (log c.f.u. ± S.D.)	
		Control animals	Treated animals
$9000/7 \times 10^5$	300	7·0 ± 0·19 (3)†	3·13 ± 1·45 (6)
$B42/2 \times 10^6$	300	5·87 ± 0·10 (2)	5·84 ± 0·25 (5)
$KH/1 \times 10^6$	300	5·89 ± 0·09 (2)	4·36 ± 0·38 (7)
$KH/1 \times 10^4$	2000	5·06 ± 0·45 (3)	< 2·0–2·47 (12)

* For 3 days following the bacterial challenge. The animals were killed 24 h after the last dose.
† Number of animals given in parentheses.

analysed 1 week after the last dose of BSS, they were similar for both the treated and control animals, approximately 10^5–10^6 per animal.

DISCUSSION

On the basis of *in vitro* studies of the activity of bismuth subsalicylate against ten *C. jejuni* strains in the present study, the organism does not appear to be very susceptible to the agent. Two strains were inhibited with a concentration of 450 μg/ml and eight strains with a concentration of 900 μg/ml. Recently Cornick and colleagues [8], using an agar dilution method, tested the activity of BSS against a broad range of intestinal pathogens and commensals (for a total of 155 bacterial strains). No *C. jejuni* strains were included in that study, however. According to the results of Cornick and colleagues [8], the MIC_{90}s of ETEC, salmonella, and *Clostridium difficile* were 4096, 2048, and 128 μg/ml, respectively. *C. difficile* was the most sensitive of the strains studied. On the other hand, *Helicobacter pylori*, a campylobacter-like organism harbouring in the gastric epithelium of humans, is very susceptible to BSS, the range of MICs being 2–32 μg/ml [9]. Various bismuth salts have been used successfully in clinical trials for the eradication of *H. pylori* from the gastric epithelium [10]. Based on the

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Table 4. *Prevention of the growth of* C. jejuni *in the intestine of infant mice treated with bismuth subsalicylate (BSS) before and after the oral challenge with bacteria*

Bacterial strain	Daily dose of BSS per animal (μg)*	Bacterial counts (log c.f.u.)			
		Control animals		Treated animals	
		Small intestine	Large intestine	Small intestine	Large intestine
KH	2000	3·47 ± 0·50	6·0 ± 0·40 (6)†	< 2·0‡	< 2·0 (10)
B6	2000	6·0 ± 0·75** (2)		< 2·0 (7)	
B42	300	4·16 ± 1·18	5·32 + 0·78 (2)	2·7 ± 0·16	2·86 + 0·60 (4)
B42	300	6·25 ± 0·15 (6)		2·87 ± 0·73	< 2·0–4·2 (10)
9000	300	6·56 ± 0·41 (3)		< 2·0–3·39	3·0 ± 11 (7)
12650	300	5·91 ± 0·38 (6)		3·16 ± 0·64 (12)	

* Two days before and 2 days after the bacterial challenge (10^4–10^5 c.f.u. per animal). A dose was also given on the day of the challenge.
† Number of animals. ‡ Sensitivity of the method, log 2·0. ** Small and large intestine together.

above results, *C. jejuni* seems *in vitro* to be more susceptible than ETEC, but less susceptible than *H. pylori*, to the activity of BSS. However, the results of different antimicrobial sensitivity studies cannot be directly compared, e.g. due to the poor solubility of BSS. The mechanism of the antimicrobial activity of bismuth salts against bacteria is not well understood. In the *in vitro* studies, a focal accumulation of particulate bismuth complex occurred under the cell wall, but no morphological changes were detected in *H. pylori* [11]. On the other hand, in the *in vivo* studies ultrastructural changes were seen in *H. pylori* in the endoscopic biopsies after treatment of patients with bismuth salts [10]. No results for other organisms were available. It is possible, however, that BSS acts in a non-specific way, preventing bacterial cell attachment to mucus or to specific receptors. Lee and co-workers [12] showed that in the mouse, campylobacters are mucus-colonizers, and do not adhere to the epithelial cells. Thus they are accessible to drugs such as bismuth compounds which are not absorbed but act locally on mucus surfaces. BSS was not shown to cause any significant changes in the total counts of intestinal flora or in the counts of specific groups of enteric organisms [13]. More research is needed to elucidate the antibacterial mechanism of BSS.

In the present study, the *in vivo* activity of BSS against *C. jejuni* was studied using infant mice challenged with *C. jejuni* in three different treatment regimens. The results provided some evidence of the *in vivo* activity of the agent. The BSS treatment was considered to be effective if the counts in treated animals were at least two log units lower than in control animals. This method has been used in the evaluation of the efficacy of vaccination in protection against colonization of *C. jejuni* in infant mice [14]. The statistical evaluation of the results confirmed the efficacy of the dosage regimens when BSS was either given as a daily pre-challenge or as a combined daily pre- and post-challenge dosage regimen. The therapeutic administration of BSS in low and high doses had a proportional effect on preventing the growth of *C. jejuni*. If the organism has already colonized, higher doses are probably needed for its eradication. In the third regimen, which represents the more typical dosage regimen used in the prevention of traveller's diarrhoea, both low and high doses prevented the growth of *C. jejuni*. These results indicate that the role of BSS has a prophylactic rather than therapeutic effect.

The infant mice model has been extensively used, e.g. in colonization [15, 16], pathogenesis [17], and immunity, [14, 18] studies of campylobacter infection. These studies have shown that the animals are colonized from stomach to colon, and that colonization lasts at least for 2 weeks. The highest numbers of bacteria are found in the caecum and colon. In the present study as well, the highest counts of campylobacters, ranging from 10^5 to 10^7, were in the distal part of the intestine in control animals. The growth inhibition of campylobacters was effective in both the proximal and distal part of the intestine. In human infection the distribution of campylobacters in the intestine is not known, but the infection appears most commonly to involve the terminal ileum and colon [19].

BSS is not absorbable, and its antimicrobial effect was shown to last only for the period of treatment. One week after the last dose, there were no differences in the counts between the treated and control animals. Similarly, BSS treatment effectively removes *H. pylori* from the gastric mucosal surface, but relapses are

frequent after the cessation of treatment [10]. The consequence of the above results for the use of BSS as a preventive treatment for traveller's diarrhoea caused by *C. jejuni* is that BSS should be continued for some days after return.

BSS has been shown to prevent traveller's diarrhoea by decreasing the occurrence of diarrhoeal symptoms as well as by acting directly on the causative agents. In studies in which the enteropathogens were investigated, the stool specimens of people treated with BSS were less commonly positive for shigella, salmonella or enteropathogenic *E. coli* than were the stool samples of the placebo group [5, 6]. In these studies, however, campylobacter examination was not included. The present *in vitro* sensitivity studies, as well as the *in vivo* studies with infant mice, indicate that BSS might be effective in the prevention of traveller's diarrhoea caused by *C. jejuni*.

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