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The Effect of Ceftriaxone on the Anaerobic Bacterial Flora and the Bacterial Enzymatic Activity in the Intestinal Tract

Summary: The normal flora of the intestinal tract, mainly consisting of anaerobic bacteria, protects the host against colonization by pathogenic microorganisms. Antimicrobial treatment with ceftriaxone may influence the colonic microflora and as a consequence, the protective effect. Ten healthy volunteers received 1 g of ceftriaxone intramuscularly for five days. This resulted in a significant decrease ($p<0.05$) of the mean cultural counts (± SEM) of total anaerobes from 10.67 (0.11) (prior to treatment) to 9.02 (0.45) and 8.97 (0.46) at days 3 and 5, respectively (during treatment). After treatment (days 10 and 15–19), the cultural counts of anaerobes returned to 10.17 (0.16) and 10.44 (0.18), respectively. Bacterial enzymes may serve as an indicator of protective microflora. β- aspartylpeptidase and deoxycholate hydrolase activity was determined in faecal supernatants of the volunteers and compared with anaerobic culturing. Both enzymatic activities show a significant correlation with the total number of anaerobes present at day 3 of ceftriaxone treatment. At day 5 and 8 only β-aspartylpeptidase showed significant correlations with cultural counts of total anaerobes, *Bacteroides* spp. or bifidobacteria. At day 15 to 19 (ten to 14 days after treatment) β-aspartylpeptidase showed only a significant correlation with the number of *Bacteroides* spp. This indicates that changes in the indigenous bacterial flora during and shortly after treatment with ceftriaxone can be monitored by determination of β-aspartylpeptidase. Recovery of the intestinal flora is difficult to assess in this manner.

Zusammenfassung: *Einfluß von Ceftriaxon auf die anaerobe Flora und die bakterielle Enzymaktivität im Intestinaltrakt.* Die normale Flora des Intestinaltraktes besteht vorwiegend aus anaeroben Bakterien und schützt den Wirt gegen eine Kolonisation durch pathogene Mikroorganismen. Eine antimikrobielle Therapie mit Ceftriaxon kann die Mikroflora des Dickdarms beeinträchtigen und damit auch deren protektiven Effekt. Zehn gesunde Probanden erhielten fünf Tage lang 1 g Ceftriaxon intramuskulär appliziert. Dies führte zu einer signifikanten Abnahme der mittleren Koloniebildnerzahlen von 10,67 (SEM ± 0,11) vor Applikation auf 9,02 (± 0,45) nach drei und auf 8,97 (± 0,46) nach fünf Tagen ($p<0,05$). Nach zehn und 15 bis 19 Tagen im Anschluß an die Antibiotikagabe kehrten die Anaerobier-Koloniebildnerzahlen auf 10,17 (± 0,16) bzw. 10,44 (± 0,18) zurück. Bakterienenzyme können als Indikator für die protektive Mikroflora dienen. In Überständen von Stuhlproben der Probanden wurden β-Aspartylpeptidase und Desoxycholat-Hydrolase bestimmt und mit den Anaerobier-Kulturen verglichen. Zwischen den Aktivitäten beider Enzyme und der am Tag 3 gemessenen Anaerobier-Gesamtzahl fand sich eine signifikante Korrelation. Am Tag 5 und Tag 8 zeigte nur die β-Aspartylpeptidase eine signifikante Korrelation mit den Gesamt-Koloniebildnerzahlen der Anaerobier sowie mit den Zahlen von *Bacteroides* spp. oder Bifidobakterien. An den Tagen 15 bis 19 (zehn bis 14 Tage nach Antibiotikagabe) bestand nur zwischen der Zahl von *Bacteroides* spp. und β-Aspartylpeptidase eine signifikante Korrelation. Nach Behandlung mit Ceftriaxon lassen sich folglich Veränderungen der bakteriellen Flora kurzfristig durch Bestimmung der β-Aspartylpeptidase erfassen, weniger gut aber die Erholung der Darmflora.

Introduction

The normal flora of the digestive tract, mainly consisting of anaerobic bacteria, protects the host against colonization by pathogenic microorganisms from the environment. This protective effect of the indigenous microflora is called colonization resistance, [1–3]. A substantial decrease in the number of anaerobic bacteria may affect the level of colonization resistance, resulting in an increased infection risk. As a consequence of the large number of different anaerobic bacteria in the intestinal tract, a large variety of bacterial enzymes are present. Any antimicrobial treatment which disturbs the protective microflora will also affect the bacterial enzymatic activity. Bacterial enzymatic activity may then serve as an indicator of the level of colonization resistance. Ceftriaxone, a third generation semisynthetic cephalosporin [4,5], is excreted into the bile to a considerable extent (11–65%) and may then influence the colonic microflora [6–10]. In the

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present study, the relationship between two bacterial enzymatic activities (β-aspartylpeptidase and taurodeoxycholate hydrolase) and the cultural counts of the total number of anaerobic bacteria, bifidobacteria and *Bacteroides* spp. was investigated in faecal supernatants of ten volunteers treated with ceftriaxone.

Materials and Methods

Volunteers: Faecal samples were collected from 29 healthy adult volunteers who did not receive antimicrobial drugs in the four weeks prior to collection of the faecal samples. These samples were used to determine whether β-aspartylpeptidase and taurodeoxycholate hydrolase activity was generally present in faeces. Ten other healthy adult volunteers received 1 g of ceftriaxone intramuscularly for five consecutive days. Faecal samples were collected at day 1 prior to antibiotic treatment, day 3 and 5 during treatment and three to 14 days after treatment. The study involving antimicrobial treatment was approved by the Medical Ethical Committee of the University Hospital of Groningen.

Faecal enzyme preparation: Faecal suspensions (25%, w/v in water containing 0.1% triton X-100) were prepared by homogenization with an ultra-turrax and subsequent centrifugation. After centrifugation at 9000 x g for 10 min, supernatants were centrifuged for 60 min at 31,000 x g. The resulting supernatants were dialyzed against demineralized water. The retentate was used as bacterial enzyme preparation.

β-Aspartylpeptidase and taurodeoxycholate hydrolase assay: Eighty µl of the dialyzed faecal supernatant was added to 80 µl 0.04 M sodium phosphate, pH 7.2, containing 2.4 mM taurodeoxycholate (Sigma Chem., St. Louis, MO), 2.4 mM β-aspartylleucine (β-Asp-Leu, Bachem, Bubendorf, Switzerland) and 0.5 mM zinc sulphate. Sixty µl of this mixture was subjected to high-voltage paper electrophoresis at pH 3.5 [11] after 20 h incubation at 37°C. The amount of aspartic acid liberated after cleavage of β-Asp-Leu and the amount of taurine liberated after cleavage of taurodeoxycholate were taken as a measure of β-aspartylpeptidase and taurodeoxycholate hydrolase activity, respectively. The amount was determined semi-quantitatively by comparing with the intensities of known amounts of aspartic acid and taurine after staining with ninhydrin. The enzyme activity in the faecal supernatant of each individual was considered to be 100% at day 1 prior to antibiotic treatment. As a control, 80 µl of the faecal supernatant of each volunteer with 80 µl of 0.04 M sodium phosphate was incubated at 37°C.

Anaerobic culturing: Stool samples were processed as described by *Holdeman* et al. [12]. Processing for microbiologic studies, isolation, and identification of anaerobes proceeded as described earlier [13].

Statistical analysis: The relationship between β-aspartylpeptidase, taurodeoxycholate hydrolase, and the cultural counts of anaerobic bacteria as well as bifidobacteria and *Bacteroides* spp. on different days (3, 5, 8 and 15 to 19) during and after antimicrobial treatment was investigated. Spearman rank correlation coefficients were tested for significance ($P<0.05$) [14].

Results

Incubation of the faecal enzyme preparations of 29 volunteers showed that a large interindividual variation (fivefold differences) existed in the amount of aspartic acid and taurine liberated after cleavage of β-Asp-Leu and taurodeoxycholate, respectively. The faecal supernatant of one volunteer did not contain β-aspartylpeptidase and in that of one other volunteer no taurodeoxycholate hydrolase activity could be detected. However, in all 29 faecal samples, either β-aspartylpeptidase or taurodeoxycholate hydrolase activity could be detected. Therefore this simultaneous assay of both enzyme activities could be applied to monitor faecal supernatants from ten other volunteers who were treated with ceftriaxone for five days. Ceftriaxone treatment resulted in a significant decrease ($P<0.05$) in the cultural counts (± SEM) from 10.67 (0.11) at day 1 (prior to treatment) to 9.02 (0.45) and 8.97 (0.46) at days 3 and 5, respectively. The cultural counts of total anaerobes returned to pretreatment level and were 10.17 (0.16) and 10.44 (0.18) at day 10 and days 15–19, respectively. The cultural counts in the faeces of each volunteer together with data on the occurrence of diarrhea and presence of *Clostridium difficile* are given in Table 1. In the faeces of some volunteers there was a large decrease in cultural counts of total anaerobes (e.g. volunteer 8) while in the faeces of other volunteers these counts remained at a constant high level (e.g. volunteer 1). This might be the result of differences in excretion [6–9] or inactivation by bacterial enzymes present in the faeces [10,14].

An example of the effect of ceftriaxone on the total number of anaerobic bacteria and the two enzyme activities of volunteer 8 is shown in Figure 1. After the onset of ceftriaxone treatment, anaerobe cultural counts dropped in this volunteer from 10.76 to 7.48 on day 3 and to less than 7.00 on day 5. The taurodeoxycholate hydrolase activity follows this pattern, while the β-aspartylpeptidase activity decreases more slowly. A

Table 1: Cultural counts of total anaerobes in the faeces of ten volunteers who received ceftriaxone i. m. during five consecutive days. Day 1 (prior to treatment); days 3 and 5 (during treatment); days 8, 10 and 15-19 (after treatment). *Clostridium difficile* present, +; loose stools, ±; diarrhea, +

Volunteer	Cultural counts of total anaerobes Days 1	3	5	8	10	15–19	*Clostridium difficile** at day (d)	Diarrhea/loose stools
1	10.34	10.23	10.36	10.52	10.26	11.00		±
2	10.49	< 7.00	< 7.00	6.97	10.11	10.54		±
3	10.30	7.30	< 7.00	< 7.00	9.26	10.68		±
4	10.52	9.90	10.18	9.51		10.46		±
5	10.53	10.52	10.26		10.30	9.89		±
6	10.67	10.53	10.43		10.23	10.23	+(d 5)	+(d 6,7)
7	11.42	9.48	9.04		10.40	10.53	+(d 1,7)	±
8	10.76	7.48	< 7.00	< 7.00	9.92	10.93		±
9	10.61	9.85	9.66	8.59	10.86	10.65		±
10	11.08	7.86	8.77		10.60	10.63		±

*, all toxin negative.

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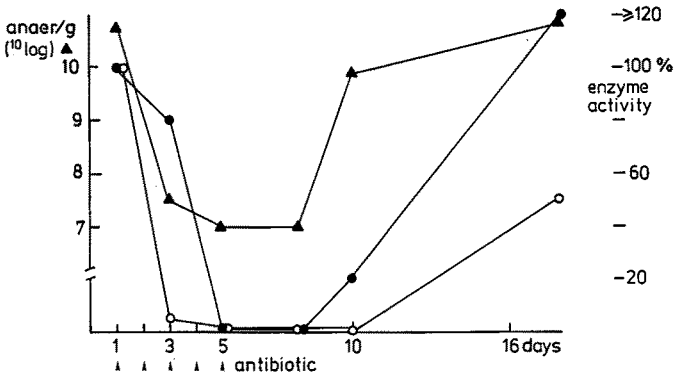

Figure 1: The effect of ceftriaxone treatment during five consecutive days (indicated by arrows) on the total number of anaerobic bacteria (^{10}log) per gram of faeces (▲), the β-aspartylpeptidase activity (●) and the taurodeoxycholate hydrolase activity (○) of volunteer 8. The enzyme activity at day 1 prior to antibiotic treatment was taken as 100%.

Table 2: Relationship between the presence of β-aspartylpeptidase and taurodeoxycholate hydrolase activity and the largest decrease (in ^{10}log) in cultural counts of the total number of anaerobes, *Bacteroides* spp. and bifidobacteria during and after treatment with ceftriaxone (compared to day 1 prior to ceftriaxone treatment).

Volunteer	β-Aspartylpeptidase	Taurodeoxycholate hydrolase	Decrease of total anaerobes	Decrease of bifidobacteria	Decrease of *Bacteroides*
1	+	+	−0.66	1.23	0.38
2	−	n.a.	3.52	3.24	>5.18
3	−	−	>3.30	>4.20	>3.66
4	−	−	1.01	0.40	>3.46
5	+	+	0.64	1.07	−0.91
6	+	−	0.44	2.70	1.16
7	−	−	2.38	1.95	>5.04
8	−	−	>3.76	4.87	>4.15
9	−	+	1.66	2.28	0.93
10	−	−	3.22	2.70	2.37

Enzyme activity of less than 10% is considered negative (−); enzyme activity more than 10% of the activity at day 1 (prior to antibiotic treatment) is considered positive (+); n.a., no activity prior to, during and after ceftriaxone treatment.

comparison of major changes in the colonic anaerobic flora (total number of anaerobes, bifidobacteria and *Bacteroides* spp.) and the bacterial enzymatic activity in the faeces of each volunteer is shown in Table 2. The individual enzymatic activities at day 1 prior to antibiotic treatment were taken as 100%. Bacterial enzymatic activity was considered to be present or positive (+) during and after ceftriaxone treatment when it was more than 10% of the activity at day 1 (prior to antibiotic treatment). A large decrease in the anaerobe cultural counts (2.38 or more) is reflected by a lower bacterial enzymatic activity. However, a small decrease in the total number of anaerobes may be accompanied by a large decrease in an enzyme producing species. For example, in

Table 3: Spearman rank correlation coefficients of the decrease in cultural counts of total anaerobes, *Bacteroides* spp., bifidobacteria at day 3 and 5 (during ceftriaxone treatment) and at day 8 and 15 to 19 (after treatment) compared to day 1, and the corresponding β-aspartylpeptidase and taurodeoxycholate hydrolase activity (% of activity at day 1).

Day	β-Aspartylpeptidase		Taurodeoxycholate hydrolase
3	total	−0.68 (P<0.05)	−0.75 (P<0.05)
	Bacteroides	−0.74 (P<0.02)	
5	total	−0.87 (P<0.01)	
	bifidobacteria	−0.84 (P<0.01)	
8	total	−0.90 (P<0.05)	
	Bacteroides	−0.96 (P<0.02)	
15 to 19	*Bacteroides*	−0.69 (P<0.05)	

the faeces of volunteer 4, cultural counts of total anaerobes decreased from 10.52 to 9.51, which suggests that no major change had occurred in the flora. However, *Bacteroides* cultural counts dropped from 8.46 to less than 5.00. Apparent exceptions: i) the taurodeoxycholate hydrolase activity in the samples of volunteer 6 was low while the β-aspartylpeptidase activity remained unaffected and ii) the β-aspartylpeptidase activity in the samples of volunteer 9 was low while the taurodeoxycholate hydrolase activity was not affected. However, examination of these activities on each sampling day show that the above-mentioned activities were low but never completely negative.

Statistical analysis of the data on a particular day more clearly shows the relationship between the colonic microflora and the bacterial enzymatic activities. In Table 3, the Spearman rank correlation coefficients are listed. A significant negative correlation between the difference in cultural counts of total anaerobes (compared to day 1) and both enzyme activities (% activity compared to day 1) was only found on day 3 of ceftriaxone treatment. More correlations were found between the faecal flora (especially the number of *Bacteroides* spp. and total anaerobes) and the β-aspartylpeptidase activity.

Discussion

It has been reported earlier that a considerable number of strains of anaerobic bacteria produce β-aspartylpeptidases [16]. Similarly, it is well documented that hydrolysis of conjugated bile acids is the result of a specific enzymatic activity of many intestinal bacteria [17]. Large disturbances in the intestinal anaerobic flora therefore could have a marked effect on intestinal bacterial enzymatic activity. A decrease in the activity may indicate disturbance of the protective microflora and thus in the level of colonization resistance. The results show that at day 3 of ceftriaxone treatment both enzymatic activities show a significant correlation with the total number of anaerobes (Table 3). Only β-aspartylpeptidase activity

showed a significant correlation with changes in the total number of anaerobes during a longer period (day 3, 5 and 8).

Recovery of the flora is more difficult to assess. Although the β-aspartylpeptidase activity shows a significant correlation with the number of *Bacteroides* spp. ten to 14 days after antibiotic treatment, this activity did not always return to pretreatment level within the posttreatment observation period. This was also found for the taurodeoxycholate hydrolase activity. This could imply that after treatment other bacteria, not able to produce these enzymes, may have become the major constituents of those individual microfloras.

In conclusion, the results show that changes in the faecal concentration of two bacterial enzymes can be determined simultaneously by high-voltage paper electrophoresis. These changes, in particular β-aspartylpeptidase, reflect changes in the indigenous bacterial flora during (day 3 and 5) and shortly after (day 8) antimicrobial treatment. The intactness of the flora 10 to 14 days after treatment is difficult to assess in this manner, since during the process of recovery of the intestinal flora, the enzymatic activity may be suppressed or the ability to produce these enzymes may be lost.

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