Fecal Steroids in Diarrhea: IV. Cholera

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ABSTRACT

Fecal bile acid and neutral sterol patterns were studied in eight healthy adult volunteers who were challenged with *Vibrio cholerae* classical Ogawa 395 strain in the course of vaccine development studies. Bacterial 7α-dehydroxylation of cholic and chenodeoxycholic acids was not altered during experimentally induced cholera diarrhea, despite the fact that fecal weight in g/day (wet wt) was increased greatly during diarrhea (1913 ± 390 vs 161 ± 11 in controls, $p < 0.005$). Consistent with the findings on bile acids, no significant changes in the production of coprostanol, epicoprostanol, or coprostanone were observed although the percentage of unmodified cholesterol was increased during the diarrheal episode (20.7 ± 3.3% vs 11.9 ± 2.3, $p < 0.02$). Total concentrations of both bile acids and cholesterol in mg/g of feces (wet wt) were decreased considerably as a result of diarrhea). However, total bile acid and neutral steroid excretions in mg/kg/day in subjects with and without diarrhea do not appear to be different. Intestinal transit times, measured in eight subjects by the use of carmine red dye, were found to be shortened in diarrhea (5.8 ± 1.1 hr vs 23.4 ± 4.1 hr in controls, $p < 0.001$). The results from this study are similar to those observed in experimentally induced travellers' diarrhea associated with toxigenic *Escherichia coli*, but they are in striking contrast to the changes in gastrointestinal steroid metabolism observed in acute shigellosis, an invasive intestinal infection.
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Extensive changes in fecal bile acid (BA) and neutral steroid (NS) patterns were observed in a previous investigation conducted in normal volunteers who were infected with *Shigella flexneri* (1). Bacterial degradation of fecal steroids was found to be reduced despite the fact that total bile acid and neutral sterol concentrations were diluted. In a similar investigation (2), volunteers who had received a challenge of enterotoxigenic *Escherichia coli* did not have changes in the gastrointestinal metabolism of bile acids or neutral steroids. This contrasting pattern of response to diarrheal etiologic agents suggested specific alterations in bile acid metabolism associated with the etiology of diarrhea. To test this conclusion, the observations were extended to a study of volunteers challenged with *Vibrio cholerae*. The mechanism of diarrhea produced by both *E. coli* and *V. cholerae* is mediated through enterotoxin-induced stimulation of adenylate cyclase within mucosal enterocytes of the small

bowel. The resultant intracellular accumulation of cyclic AMP results in net intestinal secretion in the absence of bacterial invasion. This stands in contrast to diarrhea mediated by *S. flexneri* which requires overt mucosal invasion, usually of the large bowel mucosa. The purpose of this investigation was to determine whether *V. cholerae*, the prototype of small bowel secretory diarrhea, would produce the same pattern of bile acid secretion as that produced in the milder disorder associated with *E. coli* (3), confirming the conclusion made in the previous publication that the mechanism of diarrhea is reflected by fecal sterol pattern.

MATERIALS AND METHODS

Bacteriology

V. cholerae classical Ogawa 395 strain produces an enterotoxin and causes a profuse watery diarrheal syndrome in volunteers that is typical of cholera. This strain was fed to volunteers as part of a long-term program to develop vaccines against cholera (4,5).

Volunteer Studies

Volunteers were healthy adults (5 males, 3 females), ranging in age from 19 to 32 yr. Studies were carried out under quarantine in the Isolation Ward of the Center for Vaccine Development located within the University of Maryland

Abbreviations in Fig. 1: C = cholic, CDC = chenodeoxycholic, DOC = deoxycholic, LC = lithocholic, Keto A = ketohydroxy bile acids including 7-ketodeoxycholic, 12-ketolithocholic, 7-ketolithocholic, and 3, 12-diketocholanic acids, UNIDENT = unidentified bile acids, ISODOC = isodeoxycholic, URSODOC = ursodeoxycholic. Abbreviations in Fig. 2: CH = cholesterol, CO = coprostanol, EPICO = epicoprostanol, COO = coprostanone, and UNIDENT = unidentified endogenous neutral steroids.

Hospital. The methods of medical screening, clinical surveillance and care of the volunteers, informed consent, preparation of the *V. cholerae* inoculum and bacteriologic culture techniques have been previously described (4,5). Control stool specimens were collected either prior to challenge or four weeks after recovery from diarrhea. Each subject was fasted 1-½ hr before and after oral inoculation with vibrios. In order to neutralize gastric acid, thereby ensuring occurrence of diarrhea with a smaller inoculum, 2 g $NaHCO_3$ was added to 150 ml distilled water and the volunteers drank 120 ml. The inoculum (10^6 live organisms of *V. cholerae* classical Ogawa 395 strain) was added to the remaining 30 ml and was ingested 1 min later. All subjects developed cholera diarrhea within two days postinoculation (4,5). Duplicate fecal samples collected during diarrhea, but prior to medical treatment, were studied to determine the effect of diarrhea on steroid metabolism. Methods for collection of specimens have been described previously (1). Intestinal transit times (ITT) were measured for all of the subjects prior to challenge and during diarrhea; each subject ingested 500 mg of carmine red dye, a nonabsorbable marker, that was monitored as described by Higgs et al. (6) and Dimson (7).

Steroid Analysis

Duplicate aliquots of the homogenized stools were analyzed for BA and NS. Detailed procedures of thin layer chromatography (TLC) for the separation of BA have been described elsewhere (8,9). BA were analyzed by gas liquid chromatography (GLC) according to methods described by Kuksis (10) and Yousef et al. (11). Neutral steroids were analyzed by the combined TLC and GLC method described by Miettinen et al. (12). GLC analyses were performed with a Packard Becker gas chromatograph Model 420 with dual flame ionization detectors. Chromatographic conditions and procedures have been described previously (1). 5α-Cholestane was used as an internal standard for quantitation of both BA and NS. Cholic-24-^{14}C and cholesterol-7α-^{3}H of high specific activity were added as internal recovery standards to correct for incomplete recoveries during extraction and TLC.

RESULTS

Effects of Cholera on Fecal BA

The BA profile of fecal samples collected from eight volunteers before and during infection with *V. cholerae* (but before antibiotic therapy) is shown in Figure 1. Statistical evaluation was done by Student's t-test for the paired samples. All values were expressed as (mean ± SEM)
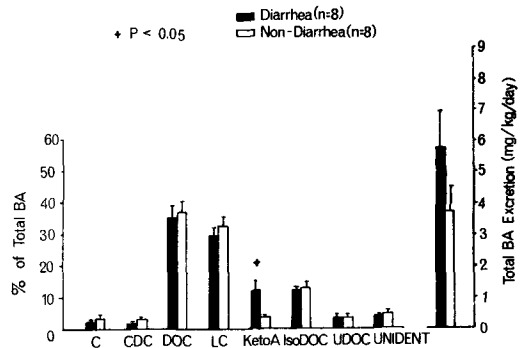

FIG. 1. Fecal bile acid profiles for eight adult volunteers challenged with *V. cholerae* classical Ogawa 395 strain. All values (mean ± SEM) are expressed as percentage of total bile acids. Total bile acid excretion is expressed as mg/kg body wt/day.

percentage of total BA in the feces so that the effect of dilution could be avoided. Fecal weights in g/day (wet wt) were increased greatly during diarrhea (1,913 ± 390 vs 161 ± 11 in controls, p < 0.005). Total BA concentrations, in mg/g feces (wet wt), were lower during the diarrheal episode (0.34 ± 0.15) than those in nondiarrheal controls of the same subjects (1.87 ± 0.52, p < 0.02). However, it can be seen from Figure 1 that there were no significant changes (p > 0.05) in the composition of BA for the eight *V. cholerae* challenged subjects, except that ketohydroxy BA was increased in diarrheal samples (11.9 ± 3.3% vs 3.5 ± 0.6%, p < 0.05). Total excretion of BA, expressed as mg/kg body wt/day, was not significantly different.

Effect of Cholera on Fecal NS

The NS profile of feces from the same eight subjects before and during cholera diarrhea, but before antibiotic treatment, is shown in Figure 2. As seen in the case of BA, total NS concentrations in mg/g feces (wet wt) were reduced greatly in diarrheal samples (0.57 ± 0.15 vs 3.97 ± 0.43, p < 0.001). In addition, the percentages of unmodified cholesterol (CH) were increased during the diarrheal episode (Fig. 2). However, no significant changes in NS metabolites, e.g., coprostanol (CO), epicoprostanol (EPICO), and coprostanone (COO), were observed. Total excretion of NS expressed as mg/kg body wt/day was not significantly different.

Sequence of Bile Acid Alteration

Figure 3 shows the sequence of BA alterations in the stool samples of one subject (J.P.) during cholera infection. The diarrhea started
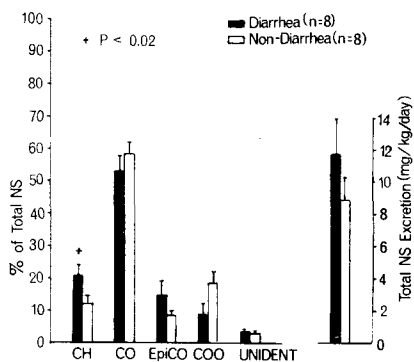
FIG. 2. Fecal neutral steroid profiles for eight adult volunteers challenged with *V. cholerae* classical Ogawa 395 strain. All values (mean ± SEM) are expressed as percentage of total cholesterol metabolites.
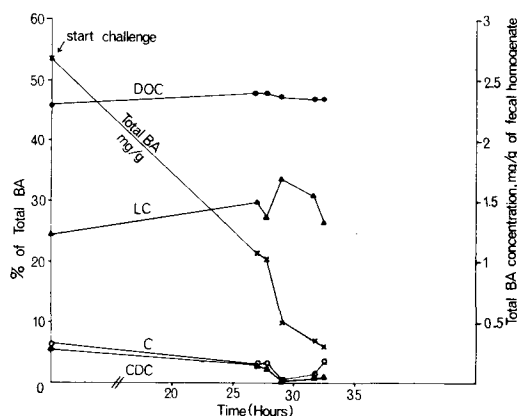

FIG. 3. Sequence of bile acid alteration in the stool samples of a subject (J.P.) with cholera. All values are expressed as percentage of total bile acid in the feces except that total bile acid concentrations are shown as mg/g fecal wet wt.

27 hr postinoculation. Four serial stools were collected within 6 hr following the initial diarrheal sample. We observed that a secondary BA, such as deoxycholic acid (DOC), remained relatively constant in all samples despite the fact that total BA concentration in mg/g feces (wet wt) decreased progressively with time elapsed after the initial episode of diarrhea. Other BA such as lithocholic (LC), cholic (C) and chenodeoxycholic (CDC) showed greater variations than DOC, but the overall pattern also appeared to be unaltered with the serial collections. This is in striking contrast with the pattern observed

in acute shigellosis where a two- to five-fold reduction in DOC and LC and a concomitant increase in C and CDC were observed within 4 hr following the initial diarrheal sample (1).

Intestinal Transit Time (ITT)

ITT (or strictly speaking, the mouth-to-anus transit time) of eight subjects in this study, as measured by the use of a carmine red marker, were found to be 5.8 ± 1.1 hr during the diarrhea associated with *V. cholerae*. The corresponding values before challenge were 23.4 ± 4.1 hr for the same subjects ($p < 0.001$). No clear relationship was noted among ITT, percentage of each BA or NS, and fecal mass (wet wt) (data not shown).

Comparison of Cholera with Other Bacterial Diarrheas

Table 1 gives the mean change in fecal steroids between the paired nondiarrheal and diarrheal samples for adult volunteer subjects challenged with *V. cholerae*. For the purpose of comparison, the corresponding values from other adult subjects with travellers' diarrhea (TD) associated with toxigenic *E. coli* (2) and those with acute shigellosis (1) from previous studies are also listed. An analysis of variance comparison of these means indicates a significant difference between *V. cholerae* and shigella for both percentage C and CH. *V. cholerae* and *E. coli* are different significantly from shigella for 7α-dehydroxylase activity, defined as the ratio ($\times 100$) of percentage DOC/(%DOC + % C), and percentage CO. There is no statistically significant difference between the three types of diarrhea for percentages of DOC, LC, CDC, or LC/(% LC + % CDC). There is no statistically significant difference between *V. cholerae* and *E. coli* for all fecal steroids listed.

DISCUSSION

We reported previously the results of a study on fecal steroid profiles of six adult volunteers who developed TD following challenge with toxigenic *E. coli* B7A and observed that bacterial 7α-dehydroxylation of cholic and chenodeoxycholic acids was not altered (2). This is consistent with the observation that the production of coprostanol from cholesterol was not changed in the same diarrheal subjects (2). These data confirm results from our earlier report (3) that bacterial modification of BA and NS was not altered in patients experiencing TD due to *E. coli* elaborating heat-stable enterotoxin. These results in *E. coli* diarrhea, however, differ markedly from the changes in gastrointestinal steroid metabolism previously observed in acute shigellosis (1). Bacterial degradation of fecal steroids was

TABLE 1

Mean (± SEM) Change in Fecal Steroid Patterns between the Paired Diarrhea
and Nondiarrheal Control Samples for Adult Volunteer Subjects

%	Small bowel diarrhea		Large bowel diarrhea	
	V. cholerae	E. coli[a]	Shigella[b]	p Value[c]
Bile acid	(n = 8)	(n = 6)	(n = 5)	
DOC	-1.7 ± 2.6	-1.4 ± 8.9	-13.9 ± 2.5	NS
LC	-3.1 ± 3.3	-12.1 ± 3.4	-15.7 ± 4.3	NS
C	-1.1 ± 1.5	+6.2 ± 4.1	+12.6 ± 2.0<	<0.01
CDC	-1.2 ± 0.9	+2.3 ± 2.4	+5.5 ± 3.5	NS
$\frac{DOC}{DOC + C}$ × 100	+1.7 ± 4.1	-11.8 ± 8.7	-46.0 ± 7.1<	<0.001
$\frac{LC}{LC + C}$ × 100	+2.5 ± 3.1	-9.9 ± 8.2	-16.7 ± 6.2	NS
Neutral sterol				
CO	-5.6 ± 8.1	-2.2 ± 9.2	-51.7 ± 7.0<	<0.005
CH	+8.6 ± 2.8	+25.1 ± 12.5	+59.6 ± 8.0<	<0.005

[a]Taken from ref. 2. The abbreviations used are the same as those in Figs. 1 and 2.

[b]Taken from ref. 1.

[c]Differences between small bowel diarrheas (cholera and/or TD) and large bowel diarrhea
(shigella) with regard to the mean changes in fecal steroids during the diarrheal episodes
before the antibiotic treatment.

NS = not significant (p > 0.05).

found to be reduced during diarrhea associated with acute shigellosis in five volunteer subjects challenged with S. flexneri 2a (strain M42-43), despite the fact that total steroid (BA and NS) concentrations in mg/g feces (wet wt) were decreased in shigellosis to a magnitude comparable to that observed in TD associated with toxigenic E. coli (2). Specifically, the percentages of DOC and LC acids of the total BA in the feces were decreased significantly in diarrheal samples with a concomitant increase in the percentages of C and CDC acids. In addition, there was a significant reduction in coprostanol content in the feces with a concomitant increase in cholesterol in the shigella diarrhea.

In this study, we have investigated the effects of experimentally induced cholera on fecal steroid profiles of eight healthy adult volunteers. The results were found to be similar to those observed in E. coli diarrhea but not to those in acute shigellosis (see Table 1). Bacterial 7α-dehydroxylation of cholic and chenodeoxycholic acids was unchanged despite the fact ITT was shortened significantly during the diarrhea episode (Fig. 1). In addition, the percentage of cholesterol metabolites, such as coprostanol, epicoprostanol and coprostanone, was unchanged although the percentage of unmodified cholesterol was increased during the cholera infection (Fig. 2). Thus, there is a reflection of at least two distinct mechanisms of diarrhea production. One, typified by enterotoxin-producing E. coli and V. cholerae, is mediated through

a stimulation of secretion in the small bowel in the absence of mucosal invasion. The other, exemplified in shigellosis, requires invasion of intestinal mucosa, particularly of the large bowel. In shigellosis, the increase in fecal primary BA and unmodified cholesterol, with concomitant decrease in secondary BA and coprostanol, speaks for a reduced interaction between luminal sterols and colonic bacterial flora. The absence of changes in 7α-dehydroxylation of BA and the biohydrogenation of cholesterol in TD associated with toxigenic E. coli and cholera indicate no appreciable alteration in interaction between intestinal sterols and bacterial flora (Table 1). This appears to be the case, even in the presence of large fecal losses and shortened ITT in cholera. Thus, it seems evident that the contrasting profiles of fecal steroids may provide a basis for biochemical differentiation between two different mechanisms of diarrhea production, i.e., stimulation of fluid secretion vs mucosal invasion. Further studies in this area are in progress.

ACKNOWLEDGMENTS

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