Human intestinal *Bacteroides* spp. RHEIN-I and RHEIN-II capable of transforming rhein to rheinanthrone, induce rhein-dependent diarrhea in rats

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Abstract

Two rhein-metabolizing bacteria were isolated from human feces. The biochemical and morphological characteristics of both isolates were typical of *Bacteroides* spp. and named strains RHEIN-I and RHEIN-II, respectively. Rhein was effectively metabolized to rheinanthrone by both strains.

In conventional male Wistar rats, diarrhea was not induced after oral administration of rhein at a dose of 100 mg/kg (fecal water content of 71 %), in spite of severe diarrhea with sennoside B at a dose of 40 mg/kg (increase of fecal water content to 89 %). Also in germ-free rats, rhein did not induce any diarrhea. Gnotobiote rats colonized with *B.* sp. strain RHEIN-I developed diarrhea (fecal water content increased to 85 %) 11 hr after the oral administration of rhein. These findings indicate that rhein-transforming bacteria are responsible for the laxative effect associated with the ingestion of rhein and rhein containing preparations.

Key words rhein, rheinanthrone, diarrhea, *Bacteroides*, gnotobiote.

Introduction

Rhein, a hydroxyanthraquinone derivative, is of interest on account of its distribution in rhubarb and other crude drugs, which are popularly used as vegetable laxatives. As regards the biological activities, rhein was found to inhibit superoxide anion radical production from human neutrophils, and several proteases, to increase hyaluronate synthesis, and to have antibacterial effects.[1-3] Rhein is also an active metabolite of its prodrug diacerein (DAR, 1,8-diacetylrhein), a drug used recently for the treatment of osteoarthritis and bone diseases.[4] Preliminary studies indicated that orally administered DAR underwent complete deacetylation to give its active metabolite rhein in animals and humans, and that the deacetylation probably occurred in the gut.[4] When DAR was orally administered to humans, diarrhea was the frequent side effect. However, rhein showed very weak laxative activity in mice when given intracecally as well as orally and parenterally.[5, 6] Similarly, danthron (1,8-dihydroxyanthraquinone), which had been used as a laxative drug to humans, was found to have weak laxative effect in mice.[7] Previous studies indicated that, when given orally, a significant amount of rhein was absorbed from the small intestine,[8] while the non-absorbed fraction moved further down the intestine to reach the colon and might undergo reduction to rheinanthrone by the action of rhein-metabolizing bacteria. It is also reported that a considerable amount of absorbed rhein is readily transformed to the glucuronide and sulfate derivatives, which are excreted in bile and undergo enterohepatic circulation.[9] These conjugates in the bile may re-enter the intestine and be converted to rheinanthrone in the colon. De Witte *et al.*[10-12] reported that cecal content of rats showed moderate ability to convert rhein to rheinanthrone, and the reducing capacity of selected anaerobes representing man and mouse intestinal bacteria was extremely low (less than 5%). These findings led us to isolate human intestinal bacteria with

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potent ability to transform rhein to rheinanthrone, an ultimate laxative principle.

Furthermore, in order to emphasize the role of human intestinal bacteria in inducing diarrhea after oral administration of rhein, the laxative effect of rhein was investigated using conventional, germ-free and gnotobiote rats.

Materials and Methods

Apparatus : An anaerobic incubator EAN-140 (Tabai Co., Osaka, Japan) was used for anaerobic cultivation. A dual wavelength chromatoscanner CS-9300 (Shimadzu Co., Kyoto, Japan) was used for determination of azomethine derivatives produced with *N,N*-dimethyl-*p*-nitrosoaniline and anthrones. For determination of fatty acids, an automass system II benchtop quadrupole mass spectrometer (Jeol, Japan) equipped with a gas chromatograph (Shimadzu Co., Kyoto, Japan) was used under the conditions [column, GB-1 [0.25 mm (i.d.)×30 m] (J & W Scientific, USA); column temperature, 50 ℃; carrier gas, He].

Chemicals and media : Rhein was isolated from *Rheum coreanum* NAKAI according to a method described by Okabe *et al.*[13] Rheinanthrone was prepared by reduction of rhein with stannous chloride,[14] and the substance was protected from light and kept dry under atmosphere of nitrogen. The identity of these compounds was established by NMR and MS. Gelatin and oxgall were products of Difco Laboratories (Detroit, USA). General anaerobic media (GAM), BL agar and SIM were purchased from Nissui Seiyaku Co. (Tokyo, Japan). Peptone-yeast extract-Fildes solution (PYF) broth was prepared according to the procedure of Mitsuoka,[15] and used as a basal medium for sugar fermentation tests.

Fermentation tests : Using a sterile solution (0.25－1.0%) of sugars or glycosides, fermentation tests were carried out in a PYF medium according to the method of Mitsuoka *et al.*[15] and reactions furnished to pH of 5.7 or below were considered to be positive. Fatty acids obtained in PYF broth containing D-glucose were determined using gas chromatography.

Antibiotics : Ampicillin sodium, penicillin G potassium, kanamycin monosulfate, neomycin sulfate (mix of B+C), gentamycin sulfate (approx. 676 μg/mg), tetracycline hydrochloride and chloramphenicol were purchased from Nacalai Tesque Co. (Kyoto, Japan), and clindamycin and erythromycin were products of Merck Co. (Darmstadt, Germany). All antibiotics were dissolved in sterile water except chloramphenicol, erythromycin and clindamycin, which were dissolved in ethanol.

Antibiotic susceptibility testing : The minimal inhibitory concentrations (MICs) were determined by the broth dilution method.[16] After anaerobic incubation (for 18 to 24 hr at 37 ℃) of bacterial strains, serially diluted antibiotic solutions were added to each culture. A control culture containing no antibiotics was included in each set.

Preparation of a mixture of intestinal bacteria : A fresh stool sample (1 g) obtained from a healthy man was suspended in 5 ml of 50 mM K-phosphate buffer, pH 7.3. The suspension was filtered with a piece of gauze and made up to 100 ml with the same buffer. This suspension was used as an intestinal bacterial mixture.

Isolation of bacterial strains capable of transforming rhein to rheinanthrone : A 5 μl portion of 250-fold diluted intestinal bacterial mixture was inoculated on BL agar plates and cultured in an anaerobic incubator at 37 ℃ for 48 hr. Colonies were selected and picked up after 24 and 48 hr incubations, and cultured in GAM broth (5 ml) for 24 hr. Individual cultures were centrifuged at 1500×*g* for 15 min. The pellets were washed with 50 mM K-phosphate buffer (pH 7.3, 5 ml), and suspended in the same buffer (195 μl). Rhein (in 1% $NaHCO_3$ solution) was separately added to each bacterial suspension to give 4.9 μmol/ml of the compound, and incubated in an anaerobic incubator at 37 ℃ for 24 h. Forty microliters of 1% *N,N*-dimethyl-*p*-nitrosoaniline (DMPA) in pyridine were added to the culture and mixed. The mixture was then extracted with butanol saturated with H_2O (200 μl). Five microlitres of the BuOH layer were applied to a TLC plate, which was developed with $CHCl_3$-MeOH (7:3). Rhein-metabolizing activity was monitored visually by observing a bluish-green spot (R_f 0.6).

Screening of defined bacterial strains for their ability to metabolize rhein : Precultured bacterial strains (100 μl each) were individually added to GAM broth (5 ml). After incubation for 24 hr, the pellets obtained after centrifugation were screened for their metabolizing activity as mentioned above. Bacterial strains used in the present experiment are as follows. *Bacteroides fragilis* ss *thetaotus, B. fragilis* ss *vulgatus, Bifidobacterium*

adolescentis, B. angulatum, B. bifidum a E 319, *B. breve* S-2 kz 1287, *B. longum* IV-55, *B. pseudolongum* PNC-2-9-G, *B.* sp. SEN, *Clostridium butyricum, C. innocuum* ES-24-06, *C. innocuum* KZ-633, *C. perfringens* TO-23, *Enterococcus feacalis* II-136, *Escherichia coli* 0-127, *Eubacterium aerofaciens, Fusobacterium nucleatum, Gaffkya anaerobia* G-0608, *Klebsiella pneumoniae* ATCC 13883, *Lactobacillus acidophilus* ATCC 4356, *L. brevis* II-46, *L. fermentum* ATCC 9338, *L. plantarum* ATCC 14927, *L. lactis* ss *lactis, Peptostreptococcus intermedius* EBF77/25, *P. anaerobius* 0240, *Proteus mirabilis* S2, and *Veillonella parvula* ss *parvula* ATCC 10790 were kindly provided by T. Mitsuoka (The Institute of Physical and Chemical Research, Wako City). *Eubacterium* sp. A-44, *Eubacterium* sp. BAR and *Ruminococcus* sp. PO1-3 were isolated in our laboratory.

Transformation of rhein to rheinanthrone by Bacteroides spp. strains RHEIN-I and RHEIN-II : Suspensions (500 μl) of *B.* sp. RHEIN-I and RHEIN-II were separately added to GAM broth (100 ml) and incubated for 24 hr under anaerobic conditions. Individual cultures were centrifuged at 1500 $\times g$ for 15 min and the pellets were washed with 50 mM K-phosphate buffer (pH 7.3, 15 ml), and re-suspended in the same buffer (2 ml). Rhein (in 1% $NaHCO_3$ solution) was separately added to each bacterial suspension to give 4.9 μmol/ml of the compound, and the mixture was incubated under anaerobic conditions. A portion (200 μl) of the culture was taken out at intervals and performed as mentioned above. The amount of azomethine derivative of rheinanthrone was analyzed by TLC-densitometry at a wavelength of 660 nm relative to a reference wavelength of 780 nm, using a calibration line of an authentic azomethine derivative of rheinanthrone. The calibration line was linear in a range of 2.48-20 nmol/spot.

Animal treatment and sampling : Male Wistar rats (6 weeks old) and male Wistar germ-free rats (WA/Jic, 7 weeks old) were purchased from Clea Japan, Inc. (Tokyo, Japan). Conventional rats were kept at an ambient temperature of 22 to 25℃ for one week before use and then kept individually in metabolic cages before the experiments. During the experiment, water and standard laboratory food (CE-2, Clea Japan, Inc.) were freely available. Germ-free rats were individually maintained in metabolic cages and kept under specific pathogen-free (SPF) conditions. Autoclaved water and sterilized CE-2 were freely available. Four germ-free rats were infected with *B.* sp. RHEIN-I (2 ml medium) on the first and the third day to create and to establish the gnotobiote rats. A sterile solution of rhein (100 mg/kg dissolved in 1% $NaHCO_3$ solution) was orally administered on the seventh day. The same dose of rhein was orally given to two groups of conventional and germ-free rats. A solution of sennoside B (40 mg/kg, in 1% $NaHCO_3$ solution) was filtered through a sterile membrane filter (0.22 mm, Millipore Co., Tokyo, Japan), and its laxative effect was similarly examined. Sterilized water (1.0 ml) was administered to three rats as the control. Fresh feces were compulsively obtained just before and at 5, 8, 11 and 24 hr after the administration, and then their water contents (%) were determined according to the following formula: water content (%) = [(fresh feces weight − dry feces weight)/fresh feces weight] × 100, where dry feces was obtained after evaporating water of fresh feces under reduced pressure overnight. The results were expressed as mean ± S.E., and statistical significance was assessed using Student's *t*-test.

Measurement of the rhein-metabolizing ability of feces : Fresh feces (1 g) from conventional, germ-free and gnotobiote rats were obtained just before the administration of rhein and were suspended in PYF broth (5 ml). Ten microliters of a rhein solution (final concentration of 4.9 mM) were added to 190 ml of this suspension and the mixture was incubated for 24 hr at 37 ℃. DMPA (40 μl) was added to the mixture, which was then extracted with butanol saturated with H_2O (200 μl). The anil adduct formed was analyzed as mentioned above.

Results

Although a human fecal suspension could effectively reduce rhein to rheinanthrone (27% conversion after 24 hr incubation), a rat cecal suspension showed weak ability to reduce rhein (10% conversion for the same period) under anaerobic conditions. When thirty defined bacterial strains from human intestinal bacteria in our stocks were screened for their metabolizing activity, no ability of transforming rhein to rheinanthrone was observed.

Isolation of bacterial strains capable of transforming rhein to rheinanthrone from human feces.

For the purpose of isolating intestinal bacteria capable of metabolizing rhein, the human fecal suspension was inoculated to BL plates. Out of 60 colonies isolated, two isolates (strains Y and Z) showed significant ability to transform rhein to rheinanthrone after incubation for 24 h. Both isolates were strictly anaerobic, Gram-negative and non-sporeforming rods (single or in pairs). The isolates grew well in PYFG broth containing 20% bile. Both produced succinic acid as a major product from the fermentation of glucose (isobutyric and isovaleric acids were also produced by strain Z). Accordingly, these isolates were considered to belong to the genus *Bacteroides*. Strains Y and Z hydrolyzed esculin, produced gas, indol and H_2S, but did not produce pigmented colonies on blood agar plates. Strain Y fermented sugars tested, xylan,[17] esculin and salicin, whereas strain Z could only utilize arabinose, glucose and lactose (Table I). Both were resistant to aminoglycosides (MIC>250 μg/ml), a general characteristic for intestinal *Bacteroides* strains tested so far.[15, 18] Furthermore, strains Y and Z were susceptible to ampicillin, penicillin G, chloramphenicol, erythromycin and tetracycline (MIC range of 0.2-22 and 1.4-4.0 μg/ml, respectively) (Table II). On the basis of the morphological and biochemical evidence,

Table I Comparative fermentation reactions of strains Y and Z

Compound	**Strain Y**	*B. ovatus**	*B. thetaiotaomicron**	**Strain Z**	*B. splanchnicus**
Amygdalin	w	+−	+w	−	−
Arabinose	+	+	+	+	+
Cellobiose	w	+	+	−	−
Esculin	+	w+	w+	−	−
Fructose	+	+	+	−	−
Glucose	+	+	+	+	+
Glycogen	+	+−	+−	−	−
Inulin	w	w	w	−	−
Lactose	+	+	+	+	+
Maltose	+	+	+	−	−
Mannitol	w	+	−	−	−
Mannose	+	+	+	−	+
Melezitose	w	+−	−+	−	−
Melibiose	w	+	+	w	−+
Raffinose	w	+	+	−	−
Rhamnose	+	+	+	−	−
Ribose	w	+	+−	−	−
Salicin	+	+	−	−	−
Starch	+	+	+	−	−
Sucrose	w	+	+	−	−
Trehalose	w	+	+−	−	−
Xylose	+	+	+	−	−
Xylan#	+	+	−	−	−

*From Bergey's Manual of Systematic bacteriology, Vol. 1 (1986).
Symbols: −, negative reaction (pH 5.9 or above); w, weak reaction (pH 5.7-5.9);
+, positive reaction (pH 5.7 or below); +−, usually positive but may exhibit a negative reaction;
−+, usually negative but may exhibit a positive reaction. #See Ref. 17.

Table II Antibiotics susceptibility of strains Y and Z

Antibiotic	MIC (μg/mL)*	
	Strain Y	Strain Z
Aminoglycoside-type:		
Gentamycin	>250	>250
Kanamycin	>250	>250
Neomycin	>250	>250
β-Lactam-type:		
Penicillin G	22.0	2.0
Ampicillin	20.0	1.4
Macrolide-type:		
Clindamycin	1.0	>100
Erythromycin	0.2	1.4
Other-type:		
Tetracycline	4.0	1.4
Chloramphenicol	1.0	4.0

*The minimal inhibitory concentration of an antibiotic that allows no visible growth after incubation for 48 hr, determined by the broth dilution method according to Koneman *et al.*[16]

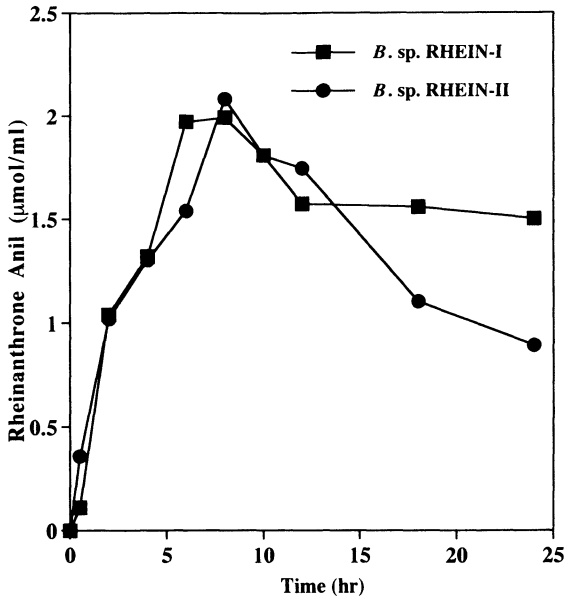

Fig. 1 Time course of conversion of rhein to rheinanthrone by *Bacteroides* sp. RHEIN-I and RHEIN-II.

strains Y and Z were preliminarily identified as *Bacteroides* sp.-like strains, named, *B.* sp. RHEIN-I and *B.* sp. RHEIN-II, respectively, with phenotypic characteristics most closely resembling those of *B. ovatus* and *B. splanchnicus*, respectively (Table I).[18, 19]

After anaerobic incubation of rhein with *B.* sp. RHEIN-I, rhein was rapidly transformed to rheinanthrone in a linear manner within 2 hr and about 40 % of rheinanthrone was formed to reach a maximum at 8 hr

Table III Transformation of anthranoids by *Bacteroides* spp. strains RHEIN-I and RHEIN-II

Compound (4.9 μmol/ml)	(μmol/ml)*	
	RHEIN-I	RHEIN-II
Rhein	2.0	2.0
8-*O*-Glucosylrhein	1.8	0.2
Aloe-emodin	1.8	1.4
Chrysophanol	1.2	0.8
Danthron	1.8	1.7
Emodin	ND	ND
Emodin 8-*O*-glucoside	ND	ND
Barbaloin	ND	ND

*Equivalent to rheinanthrone anil adduct. ND, not detected

(Fig. 1). On further incubation, the concentration of rheinanthrone was gradually decreased. This decrease may be due to decomposition of rheinanthrone and condensation of it to sennidins or other polymers. The similar pattern was also observed when rhein was incubated with *B.* sp. RHEIN-II (Fig. 1).

Aloe-emodin, chrysophanol, danthron, and 8-*O*-glucosylrhein were efficiently metabolized by both strains RHEIN-I and RHEIN-II, while emodin and its 8-*O*-glucoside resisted any transformation under anaerobic conditions (Table III).

Laxative effect of rhein after oral administration to conventional, germ-free and gnotobiote rats.

When sennoside B (40 mg/kg) was orally given to conventional rats, the rats voided wet feces even 5 hr after the administration and the laxative effect lasted for 24 hr (Fig. 2). In contrast, no significant laxation was induced by oral administration of rhein (100 mg/kg) through 24 hr. The water content of fresh feces at 5 hr after the administration was decreased to 65%, when compared with that just before the administration of rhein (fecal water content of 71%). When rhein was given orally to rats, which had been raised under germ-free conditions, no signs of laxation were observed throughout 48 hr (Fig. 3). However, in gnotobiote rats mono-associated with *B.* sp. RHEIN-I, capable of transforming rhein to rheinanthrone, severe diarrhea was induced about 11 hr after the administration of rhein and diarrhea lasted for 24 hr. The fecal water content was significantly ($p< 0.05$) increased to 85% with water-like feces.

Rhein was transformed to rheinanthrone, which was

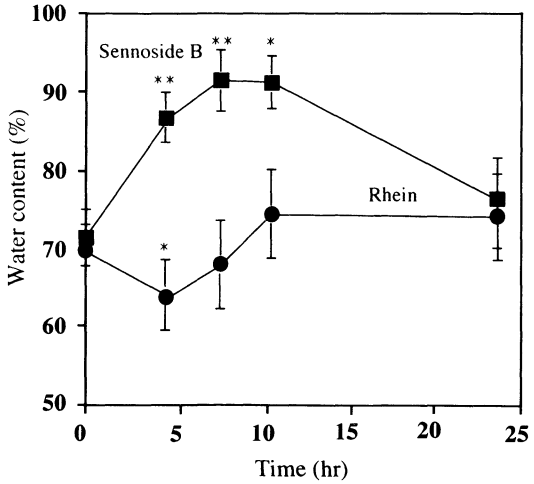

Fig. 2 Laxative effects of rhein (100 mg/kg) and sennoside B (40 mg/kg) after oral administration to conventional rats.
The water contents of feces were indicated as means ± S.E. *, $p<0.05$; **, $p<0.01$ (the water contents at the respective times *vs.* control values at time zero)

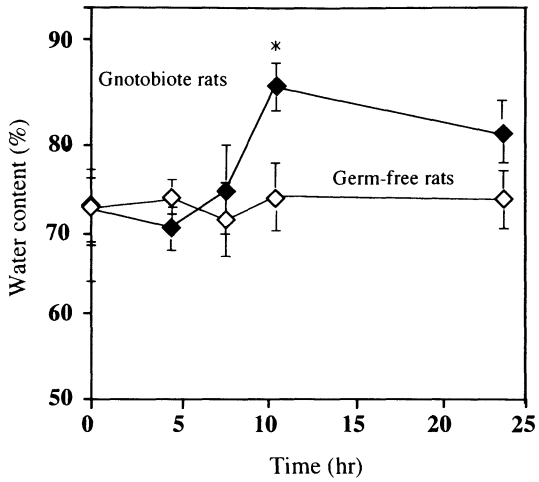

Fig. 3 Laxative effects of rhein (100 mg/kg) after oral administration to germ-free and gnotobiote rats.
The water contents of feces were indicated as means ± S.E. *, $p<0.05$ (the water contents at the respective times *vs.* control values at time zero)

detected as an adduct with DMPA, by incubation with feces from *B.* sp. RHEIN-I-infected gnotobiote rats. In contrast, no adduct was formed when rhein was incubated with feces from germ-free rats, and a trace amount of the adduct was detected in the incubates with feces from conventional rats.

Discussion

It has been reported that rheinanthrone is unstable in solutions under oxidative circumstances and gradually forms sennidins and rhein (Chart 1) or binds exclusively to bacterial memberane.[11, 12, 20, 21] However, its stable azomethine derivative formed by the immediate reaction with DMPA in pyridine prevents the consequent oxidation of rheinanthrone and makes it possible to obtain a more accurate picture of the reduction process carried out by intestinal bacteria. DMPA was directly added to the incubation mixture, and the resulting azomethine derivative was determined by TLC-densitometry.

In the present experiment, both bacterial strains, *B.* sp. RHEIN-I and RHEIN-II, rapidly and effectively con-

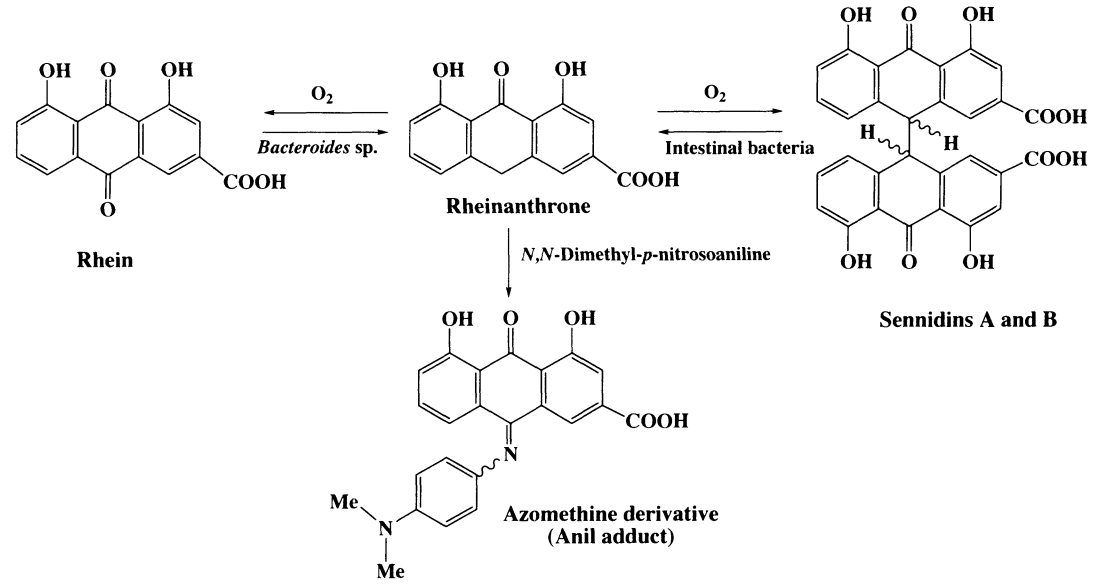

Chart 1. Conversion of rhein to rheinanthrone by *Bacteroides* sp. RHEIN-I and RHEIN-II

verted about 40% of rhein to rheinanthrone, an intestinal bacterial metabolite actually responsible for the laxative activity of rhein, after incubation for less than 8 hr. Under similar conditions, two types of strains *B. thetaiotaomicron* JCM 5824^T and *B. ovatus* JCM 5827^T from the Japan Collection of Microorganisms (JCM), the Institute of Physical and Chemical Research (RIKEN), which had been isolated from human feces, converted about 10% of rhein to rheinanthrone after incubation for 15 hr (data not shown). De Witte *et al.*[12] found that strains of *Bacteroides* and *Clostridium* species of human and mouse origins reduced rhein to rhein anthrone *in vitro*, although the conversions were less than 5% in amount after 48 hr incubation. Moore and Holdeman reported that members of the genus *Bacteroides* were among the most numerous bacteria isolated from human feces, accounting for approximately 30% of the isolates.[22] *B.* sp. RHEIN-I and RHEIN-II isolated in the present experiment were found to demonstrate significant ability to reduce rhein to rheinanthrone under our experimental conditions (Chart 1).

Taking into consideration that the oral bioavailability of rhein is very high[8] and that most of the absorbed amount is excreted in the bile as conjugates and re-enter the intestine,[9] we assume that the delay in the laxative effect of rhein in gnotobiote rats (at 11 hr), when compared with that of sennoside B (5-8 hr), was due to the enterohepatic circulation of rhein.

No laxative action was observed after oral administration of rhein to conventional and germ-free rats, but severe diarrhea was induced in gnotobiote rats infected with *B.* sp. RHEIN-I. These findings indicated that difference in the laxative effects of rhein among the rat models was attributed to the difference in the population of bacteria capable of metabolizing rhein to rheinanthrone. As a comparative study among conventional, gnotobiote and germ-free animals, we have reported on the metabolism of barbaloin where barbaloin did not induce diarrhea in germ-free and conventional rats, but did induce sever diarrhea in gnotobiote rats mono-associated with *Eubacterium* sp. strain BAR, a human intestinal bacterium capable of transforming barbaloin to aloe-emodin anthrone.[23, 24]

In conclusion, the presence of appropriate intestinal bacteria such as *B.* spp. RHEIN-I and II, and others of the genus *Bacteroides* in the human intestine should be considered to be important for the laxative effect associated with the ingestion of rhein, and the important role of intestinal bacteria in activation of natural prodrugs, sennoside and rhein, should be emphasized in connection with clinical effects of Rhei Rhizoma in traditional medicine.

和文抄録

ヒト糞便から2種のrhein代謝細菌を単離した。これらは生化学的および形態学的特徴から*Bacteroides*に属する種と判断され，RHEIN-I, RHEIN-II株と仮称した。rheinは両菌株により完全にrheinanthroneに代謝された。

通常の雄Wistarラットにおいてはrhein 100 mg/kgの経口投与で下痢が生じなかったが（糞便の水分含量は71%），sennoside Bでは40 mg/kgの用量で激しい下痢を生じた（水分含量89%）。

又，無菌ラットではrheinは何ら下痢作用を示さなかった。ところが，*Bacteroides* sp. strain RHEIN-Iを消化管内に棲息させたラットではrhein経口投与の11時間後に下痢が生じた（糞便の水分含量は85%）。

この結果は，これらrhein代謝菌がrheinやrheinを含む製剤を摂取した場合に生じる下痢作用に関与していることを示している。

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