Effect of Biotin Deficiency on the Composition of Intestinal Microflora in Osteogenic Disorder Shionogi Rats

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Summary The present study was undertaken to clarify the difference in the intestinal microflora between biotin-deficient and control groups of osteogenic disorder Shionogi rats (ODS). Thirteen four-week-old, male ODS rats weighing 70 g on average were used. Both the biotin-deficient and the control rats were fed a biotin-deficient diet, but the control rats were administered biotin (100 μ g) intraperitoneally once a week and the deficient rats were administered a comparable volume of saline. After feeding for 101 days, the cecal microflora was compared between the two groups by microbiological assay. The number of total aerobes ($p < 0.01$) and total anaerobes ($p < 0.02$) was higher in the biotin-deficient group than in the control group. The overall total anaerobes/total aerobes ratio was about 1,000 for the control group and 100 for the deficient group. This was mainly due to an increase in the numbers of the aerobic species *Staphylococcus* ($p < 0.005$) and *Enterobacteriaceae* ($p < 0.02$) in the deficient group. In the present study biotin was administered intraperitoneally to the control rats, but the composition of the experimental diet (20% egg white diet) was exactly the same for both the control and the biotin-deficient groups. Therefore, the results of the present experiment indicate that peripherally administered biotin can modify the intestinal microflora, probably via humoral changes.

Key Words: biotin deficiency, intestinal microflora, osteogenic disorder rats

Biotin deficiency can be achieved by feeding raw egg white as a source of

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avidin, a protein that binds biotin highly specifically and strongly in the gut of man and animals [1, 2]. The major characteristics of pathological symptoms of biotin deficiency are growth reduction, anorexia, depigmentation, seborrheic dermatitis, and alopecia [3, 4]. Furukawa *et al.* showed for the first time that the lumbo-sacral portion of the spine of biotin-deficient Wistar rats became arched [5], and the result was in agreement with the observation reported by Sullivan *et al.* [6]. The bone disorder caused by the biotin deficiency was attributed to a depression in collagen synthesis, which requires ascorbic acid for proline hydroxylation [7, 8].

In clinical studies Makino *et al.* [9] reported that the serum biotin level in psoriasis vulgaris and palmoplantar pustulosis patients was significantly less than in normal subjects, and it was equal to that of normal healthy infants. They also observed a high incidence of osteosclerosis and absorption of clavicular bone, especially in palmoplantar pustulosis patients. They treated patients having these syndromes with biotin and obtained successful results; i.e., 70% (35/50) of the patients with psoriasis vulgaris and 57% (17/30) with palmoplantar pustulosis showed an improvement of their dermatitis. Furthermore, they also reported several successful cases of biotin treatment of palmoplantar pustulosis patients who suffered pain in their clavicle [9]. Makino *et al.* speculated that there are several factors responsible for psoriasis vulgaris and palmoplantar pustulosis, and pointed out that intestinal microflora is one of the factors that play an important role in these syndromes because antibiotic treatment of such patients is occasionally effective. However, until now there have been few studies on the relationship between the biotin deficiency and the intestinal microflora.

Recently, the developmental mechanism of the bone disorder induced by ascorbic acid or biotin deficiency has been studied in osteogenic disorder Shionogi (ODS) rats that have a hereditary defect in their ascorbic acid-synthesizing ability [10]. The ODS rats, fed a biotin-deficient diet containing raw egg white, were afflicted with a bone abnormality including a hunch in the vertebral column [11]. However, the contribution of the intestinal microflora to biotin metabolism has not yet been thoroughly examined. We reported earlier that germ-free mice develop the symptoms of biotin deficiency much easier than their conventional counterparts [8], which means that the intestinal microflora produce biotin in the intestine and may contribute to the host nutrition. There are several reports that have shown a positive contribution of bacterially produced biotin in the distal intestine (large intestine) of the host [12–14].

The present study was undertaken to clarify the difference in the intestinal microflora between the biotin deficient and the control ODS rats, and to obtain useful information on the relationship between biotin deficiency and intestinal flora-related disorders such as psoriasis vulgaris and palmoplantar pustulosis.

MATERIALS AND METHODS

Thirteen four-week-old, male osteogenic disorder (ODS) od/od rats [10] weighing 70 g on average were generously supplied by Aburahi Laboratories, Shionogi & Co., Ltd., Koka, Japan and were divided into two groups. All animals were fed a biotin-deficient diet (20% freeze-dried raw egg white as a protein source). The composition of the experimental diet was reported previously [15, 16]. The rats were given free access to the semisynthetic diet and drinking water; and the control and the biotin-deficient groups were given an intraperitoneal injection of 0.5 ml of saline containing 100 μ g of biotin and saline only, respectively, once a week. The animals were kept in individual cages with a wire-mesh floor. Diet containers, water bottles, and cages were kept as clean as possible to prevent coprophagy.

After the rats had been fed the experimental diet for 101 days, the control and biotin-deficient animals were used for microbiological assays. The rats were sacrificed under anesthesia with ethyl ether, and the cecal contents were obtained by cutting the intestinal tract with sterile instruments. The analytical method used for assessment of the gastrointestinal microflora was essentially the same as that of Mitsuoka *et al.* [17, 18]. The cecal contents were transferred to test tubes containing transfer medium (Table 1), and the tubes were then filled with carbon dioxide gas in order to minimize the inactivation of anaerobes.

The specimens were cultured within 20 h after collection. After having been thoroughly mixed, the specimens were serially diluted from 10^{-1} to 10^{-8} with an anaerobic diluent [17]. From each dilution, 0.05 ml aliquots were spread on three non-selective agar plates: modified Eggerth-Gagnon (EG) agar (Eiken Co., Ltd., Tokyo) [18] and glucose blood liver (BL) agar (Eiken) [18] for anaerobes, and triptycase soy blood (TS) agar (BBL Microbiology System, Cockeysville, MD) for aerobes [18], as well as on 11 selective agar plates: deoxycholate hydrogen sulfide lactose (DHL) agar (Eiken) for *Enterobacteriaceae* [18], triphenyltetrazolium chloride-acridine orange-thallosulfate aesculin crystal violet (TATAC) agar (Difco Laboratories, Detroit, MI) for *Enterococcus* spp. and *Streptococcus* spp. [18], nalidixic acid cetrimide (NAC) agar (Eiken) for *Pseudomonas aeruginosa*,

Table 1. Composition of transfer medium.

Brain heart infusion (Difco)	37.0 g
Resazurin (0.1% soln, Tokyo Kasei)	0.5 ml
Agar (Difco)	1.0 g
L-Cysteine HCl·H ₂ O (Kokusan Kagaku)	0.5 g
Na ₂ CO ₃ (Wako)	4.0 g
Distilled water	up to 1,000 ml

Whole cecal content obtained from each rat was transferred to butyl rubber-stoppered tubes containing the transfer medium, and filled with carbon dioxide gas and ice-cooled until later analysis.

phenylethylalcohol egg yolk suspension (PEES) agar (Difco) for *Staphylococcus* spp. [18] (which species was further identified as coagulase-negative *Staphylococcus* [CNS] and -positive *Staphylococcus* [*S. aureus*]), potato dextrose (PD) agar (Difco) for yeasts and molds [18], neomycin-brilliant green-taurocholate-blood (NBGT) agar (Difco) for *Bacteroides* spp. [18], bifidobacteria-selective (BS) agar (Difco) for *Bifidobacterium* spp. [18], eubacterium-selective (ES) agar (Difco) for *Eubacterium* spp. [17], modified veillonella-selective (VS) agar (Difco) for *Veillonella* spp. and *Megasphaera* spp. [18], modified lactobacilli-selective (LBS) agar (BBL) for *Lactobacillus* spp. [18], and neomycin-Nagler (NN) agar (Difco) for lecithinase-positive *Clostridium* spp. [18].

Eight types of agar plates (EG, BL, NBGT, LBS, BS, VS, ES, and NN) were incubated at 37°C for 3 days in an anaerobic steel-wool jar filled with an atmosphere of oxygen-free CO₂, and the other six (TS, DHL, NAC, TATAC, PEES, and PD) were incubated aerobically at 37°C for 24 to 48 h. After incubation, each plate was examined by colony counting. The identification of the 23 bacterial groups and yeasts was made based on colonial and cellular morphology, Gram staining, spore formation, and aerobic growth. For the bacterial species identified, the bacterial number per gram of wet cecal contents was counted and converted into its logarithmic equivalent. The total viable counts were calculated from the sum of the total number of each bacterial species.

Statistical comparison of the two groups was performed by first determining population variances using the *F*-test. The two sample *t*-test was used to compare those cases of equal variance (17 cases) and Welch's correction was applied for the two cases of unequal variance.

RESULTS

A comparison of cecal microflora in the control and experimental groups is shown in Table 2. Dietary egg white, containing avidin, did not inhibit the bacterial growth in either group judging from the results of the profile and the total numbers of microorganisms found in the control and biotin-deficient groups ($10^{9.74}$ and $10^{10.47}$, respectively). A significant increase was observed in the total number of the cecal microflora in the biotin-deficient rats ($10^{10.47}$) compared with the biotin-sufficient (control) group ($10^{9.74}$, $p < 0.02$). The number of total aerobes in the biotin-deficient group ($10^{8.45}$) was higher than that in the control group ($10^{6.70}$, $p < 0.01$), and among them the *Staphylococcus* spp. counts were 1,000-fold higher ($10^{7.67}$ vs. $10^{4.69}$, $p < 0.001$) and the *E. coli* counts were 63-fold higher ($10^{8.10}$ vs. $10^{6.30}$, $p < 0.02$). The number of the total anaerobes in the biotin-deficient group ($10^{10.47}$) was also higher than that in the control group ($10^{9.74}$, $p < 0.02$); and among them the *Eubacterium* spp. counts were 13-fold higher ($10^{9.32}$ vs. $10^{8.17}$, $p < 0.005$), the *Clostridium* spp. counts, 6-fold higher ($10^{8.88}$ vs. $10^{8.10}$, $p < 0.05$), and the *Bacteroidaceae* spp. counts, 5-fold higher ($10^{10.28}$ vs. $10^{9.56}$, $p < 0.05$) than those of the biotin-sufficient rats. The overall [total anaerobes/total aerobes] ratio was

Table 2. The effect of biotin deficiency on the cecal microflora in osteogenic disorder Shionogi rats.

Cecal microflora	Mean $\pm$ SD of $\log_{10}$ (number of organisms per gram of wet cecal content)	
	Control ($n=7$)	Biotin deficient ($n=6$)
<i>Enterobacteriaceae</i>	6.30 ± 1.14 (7/7) ^a (0.04%) ^b	8.10 ± 1.07 (6/6) ² (0.43%)
<i>E. coli</i>	6.30 ± 1.14 (7/7)	8.10 ± 1.07 (6/6) ²
<i>Enterococcus</i>	6.11 ± 0.87 (7/7) (0.02%)	6.27 ± 1.09 (6/6) (0.01%)
<i>E. faecalis</i>	5.97 ± 1.09 (7/7)	6.22 ± 1.09 (6/6)
<i>Staphylococcus</i>	4.69 ± 0.86 (7/7) (0.00%)	7.67 ± 0.58 (6/6) ⁵ (0.16%)
<i>S. aureus</i>	3.62 ± 1.05 (6/7)	6.60 ± 0.96 (6/6) ⁵
CNS	4.37 ± 0.97 (7/7)	7.57 ± 0.55 (6/6) ⁵
<i>Bacillus</i>	0 (0/7)	0 (0/6)
<i>Pseudomonas</i>	0 (0/7)	5.53 (1/6)
Yeasts (YLO)	0 (0/7)	0 (0/6)
Total aerobes	6.70 ± 0.97 (0.09%)	8.45 ± 0.89^3 (0.96%)
<i>Bacteroidaceae</i>	9.56 ± 0.35 (7/7) (66.00%)	10.28 ± 0.66 (6/6) ¹ (64.58%)
<i>B. fragilis</i> group	9.27 ± 0.52 (7/7)	9.93 ± 0.63 (6/6)
<i>B. vulgatus</i>	9.28 ± 0.58 (5/7)	9.45 ± 0.82 (6/6)
<i>B. distasonis</i>	8.90 ± 0.25 (5/7)	9.52 ± 0.64 (5/6)
<i>B. uniformis</i>	8.50 (1/7)	8.60 (1/6)
<i>B. thetaiotaomicron</i>	8.07 ± 0.40 (3/7)	0 (0/6)
<i>Bifidobacterium</i>	8.20 ± 0.54 (7/7) (2.88%)	8.82 ± 0.64 (6/6) (2.24%)
<i>Eubacterium</i>	8.17 ± 0.46 (7/7) (2.69%)	9.32 ± 0.64 (6/6) ⁴ (7.08%)
<i>Peptostreptococcus</i>	8.51 ± 0.56 (7/7) (5.88%)	8.92 ± 0.64 (6/6) (2.82%)
<i>Megasphaera</i>	0 (0/7)	0 (0/6)
<i>Veillonella</i>	0 (0/7)	0 (0/6)
<i>Lactobacillus</i>	8.03 ± 0.50 (7/7) (1.95%)	7.94 ± 1.77 (5/6) (0.30%)
<i>Clostridium perfringens</i>	0 (0/7)	0 (0/6)
<i>Clostridium</i> others	8.10 ± 0.64 (7/7) (2.29%)	8.88 ± 0.52 (6/6) ¹ (2.57%)
Total anaerobes	9.74 ± 0.28 (99.91%)	10.47 ± 0.56^2 (99.04%)
Total	9.74 ± 0.28 (100%)	10.47 ± 0.56^2 (100%)

^aFrequency of occurrence expressed as the number of animals yielding the organism per total number of animals tested. ^bThe percentage of components of each genus or species.

¹⁻⁵Statistically significant at ¹ $p < 0.05$, ² $p < 0.02$, ³ $p < 0.01$, ⁴ $p < 0.005$, and ⁵ $p < 0.001$ level when compared with the control group.

about 1,000 for the control group and 100 for the biotin-deficient group.

As for the percentage of the species of microorganisms, there was an increase in *Staphylococcus* spp. (176-fold = 0.1585% : 0.0009%), *Enterobacteriaceae* spp. (11-fold = 0.43% : 0.04%) and *Eubacterium* spp. (2.6-fold = 7.1% : 2.7%) in the biotin-deficient rats. However, there was a decrease in *Lactobacillus* spp. (6-fold = 0.3% : 1.9%), *Enterococcus* spp. (4-fold = 0.006% : 0.023%) and *Peptostreptococcus* spp. (2-fold = 2.8% : 5.9%); and no change in *Bacteroidaceae* spp. (64.6 : 66.1), *Bifidobacterium* spp. (2.2 : 2.9), and *Clostridium* spp. (2.6 : 2.3).

The aerobic species, *Bacillus* and yeasts were not detected in any animals of either group, and the species *Pseudomonas* was detected in only 1 out of 6

biotin-deficient rats. The anaerobic species *Megasphaera*, *Veillonella*, and *Clostridium perfringens* were not detected in any animals of either group.

DISCUSSION

It is widely known that the composition of gastrointestinal microflora is influenced by the type of diet [19–21]. The overall counts of cecal microbes of the present experiment with ODS rats are comparable with the previously published results in which SD [22] and Wistar rats [23] were fed a commercial chow diet.

In the present study biotin was administered intraperitoneally to the control rats, but the components of the experimental diet (20% egg white diet) were exactly the same for both the control and the biotin-deficient groups. Therefore, the results of the present experiment indicate that peripherally administered biotin can modify the intestinal microflora irrespective of the dietary components. The same kind of phenomenon was also well observed when experimental rats were exposed to environmental stress such as crowding [24], heat [24], and emotional stress [25], and probably occurs via hormone-related changes in the physiology of the gastrointestinal tract [24]. In the present study, it is also considered that the microfloral changes in the cecum of rats due to biotin deficiency are probably attributable to a secondary change, such as hormonal change caused by the physiology of biotin deficiency rather than dietary components.

It has been pointed out that the post-ileal segments of the intestine are primary regions for the fermentation and vitamin synthesis in most simple-stomached animals [26]. Recently, many reports have suggested that bacterially synthesized biotin in the cecum and colon can contribute to the host's nutrition, and many researchers have emphasized the potential physiological significance of the distal intestine (large intestine) as a site of vitamin absorption [8, 12–14, 27]. Therefore, it is possible to hypothesize that the intestinal flora plays an important role in the syndromes of psoriasis vulgaris and palmoplantar pustulosis.

Ishibashi *et al.* [28] found that *Eubacterium* spp., one of the most common anaerobic bacterial groups in normal fecal flora, was almost completely lacking in the feces of a patient with acrodermatitis enteropathica. However, it dramatically reappeared at a normal level after the administration of 5-chloro-7-iodo-8-quinolinol (Entero-Vioform), with distinct improvements of all symptoms, including the low zinc level in serum.

Many microorganisms are known to be capable of biotin synthesis [29, 30]. *Bacteroidaceae* spp. and *E. coli*, which were detected as a major component of anaerobes and aerobes, respectively, in the present experiment, are also capable of biotin synthesis. The present experiment was undertaken by feeding egg white as a source of biotin-binding avidin, and thus the bacterially produced biotin in the intestine may not be absorbed, as it is already known that there is no degradation of avidin in the alimentary tract [31].

The present experiment demonstrates the necessity for identifying intestinal

flora in patients of psoriasis vulgaris and pulmopltantar pustulosis. Further characterization like the present experiment may assist the clarification of the mechanisms of these unclear diseases. As a next step of our experiment we are now planning to characterize the intestinal microflora in patients with psoriasis vulgaris and pulmopltantar pustulosis.

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