Gut Microbiota Is Not Involved in the Induction of Acute Phase Protein Expression Caused by Vitamin C Deficiency

Saiko Ikekada1, Saki Takahashi1, Norie Suzuki1, Fumiaki Hanawa1, Fumihiko Horio2 and Hiroaki Oda2

1Department of Nutritional Sciences, Nagoya University of Arts and Sciences, Nisshin 470–0196, Japan
2Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan
(Received August 22, 2019)

Summary Using rats, we previously found that vitamin C deficiency increases serum levels of interleukin-6 (IL-6) and glucocorticoid, and changes the gene expression of acute phase proteins (APP) in the liver. However, it remains unclear how vitamin C deficiency causes these inflammation-like responses. In this study, we investigated the possibility that changes in gut microbiota are involved in the induction of APP gene expression by vitamin C deficiency. ODS rats that cannot genetically synthesize vitamin C were divided into 4 groups based on the presence or absence of vitamin C or antibiotics and were raised for 15 d. Neomycin, vancomycin, and ampicillin were used as antibiotics, and 300 mg L-ascorbic acid/kg was added to the AIN93G diet. Vitamin C deficiency affected neither the wet tissue weights nor relative abundance of bacteria in the cecal contents. Antibiotic administration increased wet weights of the cecum, cecal contents, and colon, changed the relative abundance of some bacteria in the cecal contents, and decreased serum IL-6 level. However, antibiotic administration had no effect on serum concentrations of corticosterone and α1-acid glycoprotein (AGP), vitamin C concentration in the liver, and mRNA levels of haptoglobin and AGP in the liver. Therefore, disturbance of gut microbiota did not attenuate the increase in glucocorticoid level and induction of APP gene expression due to vitamin C deficiency. This suggests that gut microbiota is not involved in the inflammation-like responses caused by vitamin C deficiency.

Key Words α1-acid glycoprotein, antibiotic, ascorbic acid, corticosterone, gut microbiota, haptoglobin, interleukin-6, ODS rats, orosomucoid

Scurvy, a vitamin C deficiency, is one of the most famous nutrient deficiencies. If humans do not take vitamin C, various symptoms of scurvy appear after 45 to 80 d. That is, symptoms such as delayed wound healing, edema, bleeding from the skin, mucous membranes, and muscles, and weakening of bones, cartilage, connective tissue, etc., occur due to collagen synthesis failure (1). The detailed mechanism of how these symptoms develop due to vitamin C deficiency remains unclear. Many people believe that scurvy is a historical illness and that modern diets are high in vitamin C. However, vitamin C intake of Japanese people has decreased in recent years. In particular, the medians of vitamin C intake in the 20s, 30s, and 40s are 53, 52, and 59 mg/d, respectively (The National Health and Nutrition Survey Japan, 2017), which are well below the 100 mg/d, the recommended dietary allowance of vitamin C in the 2015 Dietary Reference Intake for Japanese. Therefore, it is important to understand early symptoms of vitamin C deficiency and chronic vitamin C deficiency.

About 20 y ago, we biochemically analyzed the serum and tissues of ODS rats fed a vitamin C-free diet for 2 wk to reveal early symptoms of vitamin C deficiency. ODS rats (genotype od/od) cannot synthesize vitamin C because of the lack of L-gulono-γ-lactone oxidase (EC 1.1.3.8), which catalyzes the terminal step of vitamin C synthesis pathway (2). No apparent deficiency symptoms such as wounds, bleeding, or growth disorders were observed after 2 wk of the deficient diet intake. However, vitamin C deficiency decreased serum concentrations of apolipoprotein A-I (ApoA-I), α2u-globulin, and albumin, and increased serum concentration of haptoglobin. All of these proteins are synthesized in the liver and secreted into the blood, and are members of acute phase proteins (APP) known to vary in expression during inflammation. Based on these facts, we have found that vitamin C deficiency changes hepatic gene expression of these APP and increases serum concentrations of interleukin-6 (IL-6), a major inflammatory cytokine, and corticosterone, a typical glucocorticoid in rats. These phenomena were not seen on day 10 of vitamin C deficiency, but always happened on day 14. However, it remains unclear how this rapid change after day 10 occurs due to vitamin C deficiency (3–5).

In recent years, the importance of the intestinal environment on the host has attracted attention. Because
gene expression of haptoglobin and α1-acid glycoprotein (AGP) is known to be induced by IL-6 (6–8), we examined a hypothesis that vitamin C deficiency changes the gut microbiota and IL-6 derived from the microbiota flows into the blood, thus changing the gene expression of APP in the liver. In this study, we investigated whether disturbance of gut microbiota by antibiotic administration attenuates induction of APP expression due to vitamin C deficiency. Contrary to this hypothesis, antibiotic administration had no effect on the APP expression, suggesting that gut microbiota is not involved in the inflammation-like responses caused by vitamin C deficiency.

MATERIALS AND METHODS

Animal study. Male ODS (ODS/Shi Jcl-od/od) rats were purchased from CLEA Japan, Inc. (Tokyo, Japan), housed in individual wire-bottomed cages, maintained in a temperature and humidity-controlled clean room with a 12-light/dark cycle (lights on from 0800 to 2000 h), and allowed access to water ad libitum throughout. The study was approved by the Laboratory Animal Care Committee of Nagoya University of Arts and Sciences (approval number 104) and all procedures performed in accordance with the Animal Experimentation Guidelines of Nagoya University of Arts and Sciences.

Six-week-old rats were divided into 4 groups: control, −C, and AB−C groups. The control and AB groups were fed a diet containing 300 mg L-ascorbic acid/kg (9) while the −C and AB−C groups were fed a vitamin C-free diet. The composition of the vitamin C-free diet based on the AIN93-rodent diet (10) was (in g/kg): casein, 200; soybean oil, 70; drinking water for the AB and AB−C groups contained 1 mg neomycin/mL, 0.5 mg ampicillin/mL. The control, −C, and AB groups were pair-fed the amount consumed by each AB−C group during the experimental period. After a 15-d experimental period, the rats were killed by decapitation. The serum and tissues were taken and stored at −30°C. The liver for determination of mRNA level was stored at −85°C.

Biochemical analysis. Vitamin C concentration in liver was determined by the dinitrophenylhydrazine method as previously described (3). Serum concentrations of IL-6, corticosterone, and AGP were determined using commercial kits; a rat IL-6 ELISA kit (Sigma, St. Louis, MO, USA), an AssayMax™ corticosterone ELISA kit (Assaypro LLC, St. Charles, MO, USA), and a rat AGP ELISA kit (Abcam, Cambridge, UK), respectively. Hepatic mRNA levels of haptoglobin and AGP were determined by real-time polymerase chain reaction analysis according to the method described by Chijimatsu et al. (11). Gene expression was normalized to the apolipoprotein E (ApoE) mRNA level. Bacterial DNA was extracted from cecal contents and the relative abundances of gut microbiota was determined using terminal restriction fragment length polymorphism analyses by the Techno-Suruga Laboratory (Shizuoka, Japan) (12, 13).

Statistical analysis. Data are presented as means±standard error for 7 samples per group. Data were analyzed by 2-way analyses of variance (ANOVA). When the significance of F values for vitamin C effect, antibiotic effect, or interactive effect (vitamin C×antibiotic) was significant (p<0.05), the mean values were compared using Tukey’s post-hoc test. Differences were regarded as significant at p<0.05. All data were analyzed using GraphPad Prism 8 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Because growth of rats was slightly suppressed by antibiotic administration in a preliminary experiment, the control, −C, and AB groups were pair-fed the amount consumed by each AB−C group during the experimental period in this study. Growth in all groups showed little difference during the experiment (Fig. 1A), although their body weight on day 15 was slightly lower due to vitamin C deficiency and antibiotics (Table 1).

Vitamin C deficiency affected neither the wet weight of all tissues measured (Table 1) nor relative abundance of bacteria in the cecal contents (Table 2). Antibiotic administration did not affect wet weights of the kidney, adrenal gland, and thymus, but increased wet weights of the colon, cecum, and cecal contents (Table 1). Compared to the cecum of the control and −C groups, the cecum of the AB and AB−C groups was markedly enlarged and yellowish (Fig. 1B). Antibiotics changed the relative abundance of some bacteria, such as Bifidobacterium, Clostridium subcluster XIVa, and Clostridium cluster XVIII, in the cecal contents (Table 2).

Antibiotic administration lowered IL-6 concentration in the serum, but had no effect on corticosterone and AGP concentrations in the serum and vitamin C.
Antibiotics Do Not Affect APP Gene Expression in Vitamin C Deficiency

Study of gut microbiota are involved in the inflammation-like response caused by vitamin C deficiency.

We previously found that vitamin C deficiency increases serum IL-6 and glucocorticoid levels and changes APP gene expression in the liver (5). In this study, we investigated the possibility that changes in gut microbiota are involved in the inflammation-like responses caused by vitamin C deficiency.

Vitamin C deficiency affected neither the wet weight of the cecum, cecal contents, and colon (Table 1), nor the relative abundance of major bacteria in the cecal contents (Table 2). Although there was no statistically significant difference, the mean of the relative abundance of Clostridium subcluster XIVa in the cecal contents of the −C group was lower than that of the control group. In other preliminary experiments, we observed a trend toward increased relative abundance of Clostridium subcluster XIVa in the cecal contents of rats fed a diet containing excess vitamin C. Furthermore, Kato et al. reported that the relative abundance of Clostridium in stools positively correlates with vitamin C intake in humans (14). Therefore, although not observed in this study, it remains possible that differences in vitamin C intake will change the relative abundance of some gut bacteria. However, antibiotic administration markedly enlarged the cecum (Fig. 1B) and increased the wet weight of the cecum, cecal contents, and colon (Table 1). Cecal enlargement is a phenomenon often seen when antibiotics are administered to rodents, which is thought to be due to inhibition of digestion of the intestinal contents by changing the gut microbiota. Antibiotic administration also increased the relative abundance of Bifidobacterium and decreased the relative abundance

### Table 1. Initial and final body weights and wet weights of tissues and cecal contents of rats.

|                | Control | −C | AB | AB−C |            |            |            |
|----------------|---------|----|----|------|------------|------------|------------|
|                |         |    |    |      | Significance of F value |            |            |
|                |         |    |    |      | C effect | AB effect  | Interaction|
| Initial body wt (g) | 142±2  | 142±3 | 142±3 | 142±2 | ns | ns | ns |
| Final body wt (g)   | 187±4b  | 178±4a | 194±5b | 187±3ab | p=0.0490 | p=0.0437 | ns         |
| Relative tissue wt (g/kg body wt) |         |    |    |      |            |            |            |
| Liver            | 37.4±0.6b | 34.9±0.7ab | 34.4±0.8a | 34.0±0.8a | ns | p=0.0189 | ns |
| Kidney           | 9.22±0.17 | 9.49±0.09 | 9.03±0.22 | 9.41±0.13 | ns | ns | ns |
| Adrenal gland    | 0.20±0.02 | 0.21±0.01 | 0.19±0.01 | 0.21±0.01 | ns | ns | ns |
| Thymus           | 2.15±0.06 | 2.10±0.20 | 2.16±0.08 | 2.30±0.11 | ns | ns | ns |
| Colon            | 2.16±0.14bc | 2.31±0.10bc | 3.07±0.18bc | 3.21±0.37 | ns | p=0.0005 | ns |
| Cecum            | 2.36±0.20b  | 3.21±0.17a  | 5.04±0.20b  | 5.07±0.33b | ns | p<0.0001 | ns |
| Cecal contents   | 10.6±1.2a  | 12.6±0.7a   | 47.0±4.0f  | 35.5±2.1b | ns | p<0.0001 | p=0.0086 |

Values are means±SE, n=7. Data were analyzed by 2-way ANOVA. The significance of F values for vitamin C effect and antibiotic effect as well as interactive effect (vitamin C×antibiotic) is shown. When the F value was significant (p<0.05), the mean values were compared using Tukey’s post-hoc test. Labeled means in a row without a common letter differ significantly (p<0.05). AB, antibiotic; C, vitamin C; nd, not detected; ns, not significant (p≥0.05); wt, weight.

### Table 2. Relative abundances of gut microbiota in cecal contents.

| (%)                    | Control | −C | AB | AB−C |            |            |            |
|------------------------|---------|----|----|------|------------|------------|------------|
|                        |         |    |    |      | Significance of F value |            |            |
|                        |         |    |    |      | C effect | AB effect  | Interaction|
| Bifidobacterium        | nd      | 0.100±0.065 | 0.183±0.089 | 0.270±0.099 | ns | p=0.0261 | ns |
| Lactobacillus          | 31.1±4.0 | 29.7±3.4 | 35.5±3.3 | 34.5±7.9 | ns | ns | ns |
| Bacteroides            | 3.78±1.15 | 3.30±1.57 | 2.39±1.21 | 1.27±0.62 | ns | ns | ns |
| Prevotella             | 2.41±2.41 | 3.30±2.39 | 0.56±0.307 | 0.272±0.129 | ns | ns | ns |
| Clostridium subcluster XIVa | 16.5±1.8c | 11.0±2.9bc | 2.10±0.60a | 3.69±1.74ab | ns | p<0.0001 | ns |
| Clostridium cluster XVIII | 0.531±0.354 | 1.06±0.43 | nd | nd | ns | p=0.0087 | ns |
| Others                 | 46.0±2.4a | 51.5±2.5ab | 59.3±2.4ab | 59.9±5.4b | ns | p=0.0043 | ns |

Values are means±SE, n=7. Data were analyzed by 2-way ANOVA. The significance of F values for vitamin C effect and antibiotic effect as well as interactive effect (vitamin C×antibiotic) is shown. When the F value was significant (p<0.05), the mean values were compared using Tukey’s post-hoc test. Labeled means in a row without a common letter differ significantly (p<0.05). AB, antibiotic; C, vitamin C; nd, not detected; ns, not significant (p≥0.05).
of *Clostridium* XIVa and *Clostridium* cluster XVIII in the cecal contents (Table 2). These results indicated that the antibiotic administered in this study had an effect on the gut microbiota of rats.

Antibiotic administration reduced serum IL-6 levels. This result indicates that the gut microbiota may contribute to an increase in serum IL-6 concentration. However, serum concentrations of corticosterone and AGP and hepatic mRNA levels of haptoglobin and AGP were increased by vitamin C deficiency, but these were not affected at all by antibiotics. These data suggest that serum IL-6 is not directly involved in increasing serum glucocorticoid level and inducing hepatic APP expression during vitamin C deficiency. Thus, the gut microbiota does not appear to be involved in the inflammatory-like responses caused by vitamin C deficiency.

Because gene expression of haptoglobin and AGP is induced by glucocorticoid, we previously examined the effect of glucocorticoid on changes in the expression of APP due to vitamin C deficiency (5). When ODS rats with adrenalectomy were fed a vitamin C-free diet for 15 d, adrenalectomy had no effect on changes of APP gene expression caused by vitamin C deficiency. This result indicates that elevated glucocorticoids do not cause changes of APP gene expression due to vitamin C deficiency.

As described in the introduction, it is also known that gene expression of haptoglobin and AGP is induced by IL-6. Tokuda et al. showed that vitamin C deficiency for 2 wk increased endotoxin in portal vein blood, suggesting that weakening of the intestinal barrier function is involved in the inflammation-like response during vitamin C deficiency (15). Kawade et al. showed that vitamin C deficiency for 2 wk induced IL-6 gene expression in the lower part of the ileum and increased IL-6 levels in the portal vein (16). In this study, antibiotic administration lowered serum IL-6 concentration. This indicates that some IL-6 in the blood is derived from gut microbiota. At least three sources of IL-6 in the blood in vitamin C deficiency are assumed: i.e., gut microbiota in the lumen of the digestive tract, intestinal cells, and endotoxins in the blood. However, IL-6 may not be a major factor in the inflammation-like responses due to vitamin C deficiency because the hepatic gene expression of haptoglobin and AGP was induced when antibiotics significantly reduced serum IL-6 levels in this study.

In this study, we investigated the effect of gut microbiota on inflammation-like responses caused by vitamin C deficiency. Disturbance of gut microbiota by antibiotic administration did not affect serum glucocorticoid lev-

| 2-way ANOVA       | C effect | AB effect | Interaction |
|-------------------|----------|-----------|-------------|
| Vitamin C in liver| p=0.0001 | ns        | ns          |
| IL-6 in serum     | ns       | p=0.0058  | ns          |
| Corticosterone in serum | p=0.0001 | ns        | ns          |
| HP mRNA           | p=0.0001 | ns        | ns          |
| AGP mRNA          | p=0.0001 | ns        | ns          |
| AGP in serum      | p<0.0001 | ns        | ns          |

Fig. 2. Concentrations of IL-6, corticosterone, and AGP in the serum and vitamin C concentration and mRNA levels of haptoglobin and AGP in the liver. Values are means + SE, n=7. Data were analyzed by 2-way ANOVA. The significance of F values for vitamin C effect and antibiotic effect as well as interactive effect (vitamin C×antibiotic) is shown. Means not sharing a letter differ at p<0.05. AB, antibiotic; C, vitamin C; nd, not detected; ns, not significant.
Antibiotics Does No Affect APP Gene Expression in Vitamin C Deficiency

Disclosure of state of COI
The authors declare no conflicts of interest.

REFERENCES

1) Combs Jr GF. 2012. Vitamin C. In: The Vitamins, Fundamental Aspects in Nutrition and Health, 4th ed, p 233–259. Academic Press, London.
2) Kawai T, Nishikimi M, Ozawa T, Yugi K. 1992. A missense mutation of L-gulono-gamma-lactone oxidase causes the inability of scurvy-prone osteogenic disorder rats to synthesize L-ascorbic acid. *J Biol Chem* **267**: 21973–21976.
3) Ikeda S, Horio F, Yoshida A, Kakinuma A. 1996. Ascorbic acid deficiency reduces hepatic apolipoprotein A-I mRNA in scurvy-prone ODS rats. *J Nutr* **126**: 2505–2511.
4) Ikeda S, Takasu M, Matsuda T, Kakinuma A, Horio F. 1997. Ascorbic acid deficiency decreases the renal level of kidney fatty acid-binding protein by lowering the α2u-globulin gene expression in liver in scurvy-prone ODS rats. *J Nutr* **127**: 2173–2178.
5) Ikeda S, Horio F, Kakinuma A. 1998. Ascorbic acid deficiency changes hepatic gene expression of acute phase proteins in scurvy-prone ODS rats. *J Nutr* **128**: 832–838.
6) Baumann H, Morella KK, Jahreis GP, Marinkovic S. 1990. Distinct regulation of the interleukin-1 and interleukin-6 response elements of the rat haptoglobin gene in rat and human hepatoma cells. *Mol Cell Biol* **10**: 5967–5976.
7) Marinkovic S, Baumann H. 1990. Structure, hormonal regulation, and identification of the interleukin-6- and dexamethasone-responsive element of the rat haptoglobin gene. *Mol Cell Biol* **10**: 1573–1583.
8) Won KA, Baumann H. 1990. The cytokine response element of the rat alpha 1-acid glycoprotein gene is a complex of several interacting regulatory sequences. *Mol Cell Biol* **10**: 3965–3978.
9) Horio F, Ozuki K, Kohmura M, Yoshida A, Makino S, Hayashi Y. 1986. Ascorbic acid requirement for the induction of microsomal drug-metabolizing enzymes in a rat mutant unable to synthesize ascorbic acid. *J Nutr* **116**: 2278–2289.
10) Reeves PG, Nielsen FH, Fahey GC Jr. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**: 1939–1951.
11) Chijimatsu T, Umeki M, Kobayashi S, Kataoka Y, Yamada K, Oda H, Mochizuki S. 2015. Dietary freshwater clam (Corbicula fluminea) extract suppresses accumulation of hepatic lipids and increases in serum cholesterol and aminotransferase activities induced by dietary chlo- retone in rats. *Biosci Biotechnol Biochem* **79**: 1155–1163.
12) Nagashima K, Hisada T, Sato M, Mochizuki J. 2003. Application of new primer-enzyme combinations to terminal restriction fragment length polymorphism profiling of bacterial populations in human feces. *Appl Environ Microbiol* **69**: 1251–1262.
13) Nagashima K, Mochizuki J, Hisada T, Suzuki S, Shimoura K. 2006. Phylogenetic analysis of 16S ribosomal RNA gene sequences from human fecal microbiota and improved utility of terminal restriction fragment length polymorphism profiling. *Biosci Microflora* **25**: 99–107.
14) Kato I, Nechvatal JM, Dzinic S, Basson MD, Majumdar AE, Ram JL. 2010. Smoking and other personal characteristics as potential predictors for fecal bacteria populations in humans. *Med Sci Monit* **16**: CR1–7.
15) Tokuda Y, Miura N, Kobayashi M, Hoshimaga Y, Murai A, Aoyama H, Ito H, Morita T, Horio F. 2015. Ascorbic acid deficiency increases endotoxin influx to portal blood and liver inflammatory gene expressions in ODS rats. *Nutrition* **31**: 373–379.
16) Kawade N, Murai A, Suzuki W, Tokuda Y, Kobayashi M, Horio F. 2019. Ascorbic acid deficiency increases hepatic expression of acute phase proteins through the intestine-derived IL-6 and hepatic STAT3 pathway in ODS rats. *J Nutr Biochem* **70**: 116–124.