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Long-term orally exposure of dioxins affects antigen-specific antibody production in mice

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ABSTRACT

Dioxins are persistent environmental toxins that are still present in the food supply despite strong efforts to minimize exposure. Dioxins ingested by humans accumulate in fat and are excreted very slowly, so their long-term effects at low concentrations are a matter of concern. It is necessary to consider long-term, low-dose continuous administration under conditions that are as close as possible to a person's diet. In this study, we orally administered 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most common dioxin, at low doses in mice and observed the immunological effects. We found that antigen-specific (OVA) antibody production in the serum increased dose-dependently by TCDD concentrations below 500 ng/kg after long-term (10 weeks) exposure. Similar increases were seen in fecal and vaginal samples but were not significant. Th1 and Th2 lymphocyte responses, as determined by antibody and cytokine production, also significantly increased dose-dependently up to 500 ng/kg TCDD, and the Th1/Th2 balance was shifted toward Th1. These results indicate that low-dose, long-term TCDD exposure results in immunological abnormalities, perhaps by increasing antigen permeability. Different doses of dioxins may have opposing effects, being immunostimulatory at low doses (100 ng/kg/day) and immunosuppressive at high doses (500 ng/kg/day).

1. Introduction

The great variety of foods available today not only supply necessary nutrients, but also enrich our cuisine. However, many Japanese have serious concerns about food safety, especially the toxic effects of synthetic chemicals such as pesticide residues, environmental pollutants, and food additives. Environmental pollutants such as dioxins and brominated flame retardants are harmful, and emissions standards are set by the government [1]. Reports of serious health hazards caused by high-concentration pollutants produced in the past have been decreasing. However, there remain serious concerns about the toxic effects of various environmental pollutants that can still be detected in human blood, breast milk, and food [2,3].

Dioxins, as represented by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are emitted into the environment from waste incinerators and other sources, and those emitted into the atmosphere eventually accumulate in the soil while those released into the water are concentrated in the food chain [4]. Humans usually intake dioxins via food, air, or water, with about 90 % of total exposure via food, which is then absorbed by the intestinal tract and distributed to various organs [5]. When dioxins are taken into the body, they are sequestered in fats, so the rate of excretion outside the body is slow, taking about seven years for a given amount of accumulated dioxins to be reduced in half in the body [6]. Since dioxins remain in the body long-term by repeated intestinal circulation, it is very important to evaluate their intestinal toxicity.

The intestinal tract not only absorbs nutrients, but also absorbs foreign substances such as chemicals and antigens that are unintentionally ingested. Our bodies are protected from external substances by biological barriers that exist in the nose and intestinal tract. These consist of a selective xenobiotic elimination system by an epithelial cell layer and an immunological barrier against xenobiotics by immunocompetent cells. The intestinal tract is the largest biological barrier and accounts for 60–70 % of the entire immune system [7].

In mice, TCDD has been demonstrated to induce thymus involution and the suppression of both humoral and cellular immunity via aryl hydrocarbon receptor (AhR), also known as dioxin receptor [8,9].

Abbreviations: AhR, aryl hydrocarbon receptor; EROD, ethoxyresorufin *O*-deethylase; Ig, immunoglobulin; IFN-γ, interferon-gamma; IL-2, interleukin-2; IL-4, interleukin-4; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; OVA, ovalbumin; TCDD, 2,3,7,8-tetrachlorobibenzo-*p*-dioxin; TDI, tolerable daily intake.

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Previous reports of immunotoxicity of orally administered TCDD were mostly single doses (1 μg/kg) over a relatively short period of time [10–12]. However, a single-dose administration is unnatural if the route of intake of TCDD is dietary. It is necessary to study the effects of daily exposure to low concentrations and low doses of dioxins that are ingested from food and the environment. Herein, we report the immune effects of long-term ingestion of TCDD, especially on antibody production, under conditions that are as close as possible to a daily meal.

2. Material and methods

2.1. Chemicals and animals

TCDD was purchased from Cambridge Isotope Laboratories (MA, USA). TCDD was dissolved in saline containing 10 % Tween20 and 1% ethanol. All other reagents were the highest quality commercially available and obtained from Nacalai Tesque (Kyoto, Japan). Female BALB/c mice (five weeks old) were obtained from SLC, Inc. The mice were housed at 23 ± 1.5 °C with a 12-h light/dark cycle and were allowed free access to standard rodent chow and water. The animal experiments were performed according to the guidelines of Setsunan University.

2.2. Oral exposure to TCDD and OVA

Mice were orally administrated 100-μL aliquots of ovalbumin (OVA, 100 μg/mouse) or a mixture of OVA and TCDD (0-1,000 ng/kg/day) daily for 10 weeks. At week 10, serum and mucosal secretions (fecal extracts and vaginal washes) were collected. Fecal pellets (100 mg) were suspended in 1 mL of phosphate-buffered saline (PBS), and extracted by vortexing for 10 min at 4 °C. The samples were centrifuged at 3,000×*g* for 10 min, and the resultant supernatants were used as fecal extracts. Vaginal mucosa was washed with 100 μL of PBS.

2.3. OVA-specific antibody production

The titrations of OVA-specific antibody in serum, fecal extracts and vaginal washes were determined by enzyme-linked immunosorbent assay (ELISA) [13]. Briefly, an immunoplate was coated with OVA (100 μg/well in a 96-well plate) in carbonate buffer, pH 9.6, and incubated with 4% BlockAce (Yukijirushi Nyugyo Co., Tokyo, Japan) for 2 h at room temperature. After that, ten-fold serial dilutions of these samples were made in 0.4 % BlockAce diluted with distilled water, and the OVA-coated plates were incubated with serially-diluted samples for 2 h. Subsequently, horseradish peroxidase-conjugated anti-mouse IgG, IgG1, IgG2a or IgA (Southern Biotechnology, Birmingham, AL) in 0.4 % BlockAce was added to the plates, and incubated for 1 h. After washing, the OVA-specific antibodies were detected using 3,3',5,5'-tetramethylbenzidine (TMB) peroxide substrate (Thermo Scientific, Rockford, IL). The reaction was stopped by adding 2 M H_2SO_4 to each well and the results were read at 450 nm using a TriStar LB 941 microplate reader (Berthold, Bad Wildbad, Germany). End-point titer was defined as the reciprocal for the highest dilution resulting in an absorbance of 0.1 optical density at 450 nm after subtracting the absorbance values of the controls.

2.4. Cytokine mRNA level in lymphocyte by real-time reverse transcription polymerase chain reaction (RT-qPCR)

An aliquot of spleen and mesenteric lymph node (MLN) extracted from mice at week 10 was gently homogenized and passed through a mesh. After lysis of red blood cells using modified ACT buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA), lymphocytes were suspended in RPMI 1640 medium containing 10 % fetal bovine serum. The cells were cultured for 2 h at 37 °C in the absence or presence of 1 mg/mL OVA. Total RNA (1×10^6 cells/well) was isolated by ISOGEN (Nippongene, Toyama, Japan), and cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa, Kyoto, Japan). PCR reactions were performed using a KAPA SYBR FAST Universal qPCR kit (Kapa Biosystems, Boston, USA) and assayed using a Thermal Cycler Dice (TaKaRa). The oligonucleotides sequence used for RT-qPCR was shown in Table 1. The Rps8 mRNA expression was not significant at any stage, so gene expression was normalized to *Rps8*.

Table 1
Primers used for RT-qPCR.

Target Genes	Forward Primers (5'-3')	Reverse Primers (5'-3')
Rps8	TTCTGGCCAACGGTCTAGACAAC	CCAGTGGTCTTGGTGTGCTGA
IFN-γ	CGGCACAGTCATTGAAAGCCTA	GTTGCTGATGGCCTGATTGTC
IL-4	TCTCGAATGTACCAGGAGCCATATC	AGCACCTTGGAAGCCCTACAGA
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
IL-13	CCTCTGACCCTTAAGGAGCTTAT	CGTTGCACAGGGGAGTCT
IL-17	GCTCCAGAAGGCCCTCAGA	AGCTTTCCCTCCGCATTGA

2.5. Statistical analysis

Statistically significant differences in means were determined using one-way an analysis of variance (ANOVA) followed by the Dunnett multiple comparison test. *P* values of less than 0.05 were considered significant. Analysis was carried out using Prism software.

3. Results

3.1. Adverse effects of long-term exposure to TCDD

When animals receive a lethal dose of TCDD, most animals experience sudden weight loss (systemic wasting syndrome) and die several weeks after exposure [14]. To determine the dose of TCDD with no systemic wasting disease, we monitored body weights for 10 weeks. At week 10, mice exposed to TCDD at 0.5, 5, 50, 100, and 500 ng/kg showed no effects on body weight compared to vehicle-gavaged controls. Furthermore, no difference was observed in spleen/body weight ratio (Table 2) However, all mice administered a dose of 1 μg/kg died during week 2. We therefore decided to use a concentration of 500 ng/kg (high dose) or less in this study.

3.2. Long-term exposure to TCDD was facilitated Antigen specific antibody production

To clarify whether TCDD can induce antigen-specific humoral responses, we investigated its effect on antigen-specific antibody production. Mice were orally administrated a mixture of OVA, a model antigen, and TCDD every day for 10 weeks after intraperitoneal immunization with OVA and alum. The antibody titers of serum OVA-specific IgG, IgE, and fecal OVA-specific IgA were not changed between vehicle and TCDD treatment until week 10 (data not shown). Therefore, we performed experiments in the absence of adjuvant using

Table 2
Body and spleen weights in TCDD-exposed mice.

TCDD administration (ng/kg)	Body weight (g)	Spleen weight (mg)	Spleen/body weight (ratio)
0	21.3±0.56	113.3±5.21	5.07±0.31
0.5	21.1±0.27	125.4±6.10	5.28±0.22
5	21.0±0.48	120.4±4.00	5.13±0.14
50	21.1±0.07	106.7±1.68	5.00±0.19
100	21.4±0.38	129.7±3.94	5.69±0.06
500	21.3±0.30	109.6±3.42	4.99±0.12

Mice were orally administrated TCDD and OVA for 10 weeks. The body weights of the mice were measured every day, and were shown at week 10. The data are shown as means ± SE (n = 10).

mice that had not been sensitized with OVA and alum. As shown in Table 3, the antibody titers of serum OVA-specific IgG increased in a dose-dependent manner at week 10. Fecal OVA-specific IgA and vaginal OVA-specific IgA were also increased in a dose-dependent manner, but the differences were not significant. Interestingly, both OVA-specific IgG and IgA titers at 500 ng/kg were decreased compared to those at 5 and 50 ng/kg. However, serum OVA-specific IgE was not detected. The dose of TCDD used in this study did not induce systemic wasting syndrome, suggesting that TCDD directly suppresses the immune response. These results suggested that TCDD had an immunostimulatory effect at low doses.

3.3. Induction of Th1 and Th2 responses by TCDD

T lymphocytes present in gut-associated lymphoid tissue are predominantly $CD4^+$ cells of both Thl and Th2 phenotypes [15]. We next examined whether oral exposure to TCDD induced Th1- or Th2-type responses. The OVA-specific IgG1 (a Th2 response) and IgG2a (a Th1 response) responses in the serum of mice orally administrated TCDD were significantly enhanced compared to those of vehicle-gavaged controls (Fig. 1). The titers of IgG1 antibody were higher than those of IgG2a in both the control group and TCDD administration group.

To determine whether these subclass variations were due to differences in the ability to produce Th1 (IFN-γ and IL-2) or Th2 (IL-4, IL-10, and IL-13) cytokines, the mRNA levels of cytokines produced when splenocytes isolated from mice orally administrated OVA and TCDD, then restimulated with OVA, were measured. In all measured cytokines except for IL-10, dose-dependent cytokine production was observed by restimulation of OVA (Fig. 2). Similar to the previous results, a decrease in cytokine production was observed in the 500 ng/kg group. IL-17, which is related to Th17, showed similar results. Although the results of lymphocytes from MLN were not remarkable, they were almost the same as those from splenocytes (data not shown). These results suggested that oral exposure to TCDD enhances the production of various cytokines, resulting in abnormal antigen-specific antibody production.

4. Discussion

Human being take low levels of toxic compounds every day. It is important to consider long-term, low-dose continuous administration. We examined the effect of daily oral intake of TCDD on antibody production. At 10 weeks after administration, OVA-specific IgG increased in a TCDD dose-dependent manner below 500 ng/kg. Further experiments suggested that this change may be dependent on TCDD affecting various cytokines produced by lymphocytes of the spleen and MLN by antigen stimulation. Since there was no change in body and spleen weight, it is considered that the decrease in antibody titer at 500 ng/kg is not due to a systemic wasting syndrome and spleen disorder but to a decrease in lymphocytes such as T cells and B cells and a change at the molecular level such as AhR signaling pathway [14,16]. It is necessary to analyze the molecular mechanism of cytokine production based on population analysis.

Table 3
Production of OVA-specific IgG and IgA.

TCDD administration (ng/kg)	Serum IgG	Fecal IgA	Vaginal IgA
0	8.26±3.59	0.24±0.07	0.047±0.047
0.5	6.37±1.07	0.27±0.10	0.213±0.113
5	70.82±39.54	0.29±0.06	0.281±0.131
50	128.47±53.12	0.33±0.10	0.320±0.114
100	278.44±68.22**	0.37±0.11	0.350±0.139
500	17.98±4.49	0.25±0.08	0.107±0.071

Mice were orally administrated TCDD and OVA daily for 10 weeks. At week 10, OVA-specific serum IgG, fecal IgA, and vaginal IgA titers were determined with ELISA. The data are shown as means ± SE (n = 10). $^{**}P < 0.01$, compared with vehicle.

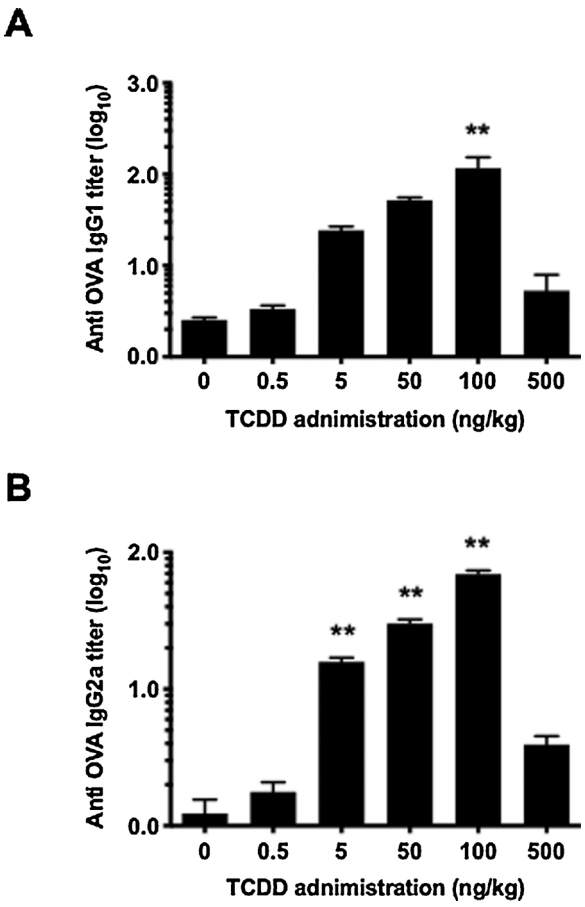

Fig. 1. Production of OVA-specific IgG subclass.
Mice were orally administrated TCDD and OVA daily for 10 weeks. At week 10, OVA-specific serum IgG1 (A) and IgG2a (B) were determined with ELISA. The data are shown as means ± SE (n = 10). $^{*}P < 0.05$, compared with vehicle.

Mucosal tissues such as the intestines contain immunocompetent cells for adaptive immunity. B and T lymphocytes form a dynamic mucosal network for the induction and regulation of secretory IgA and cytotoxic T lymphocyte responses [17]. When an antigen is administered to the mucosal surface, antigen-specific IgA is secreted not only on the mucosal surface but also on remote mucosal surfaces [13]. Although there was no statistical significant, TCDD showed a tendency to promoted OVA-specific IgA secretion on immunized sites, but not on remote mucosal surfaces such as the vaginal surface. This suggested that TCDD slightly induced mucosal immunity on the surface and further induced systemic immunity more strongly than mucosal immunity. Th1 cytokines such as IFN-γ promote isotype switching of IgM to IgG2a, whereas Th2 cytokines such as IL-4 promote isotype switching to IgG1, IgG2b, or IgG3 [18,19]. M50354, the AhR ligand, skews the Th1/Th2 balance toward Th1 dominance [20]. Our results showed that the rate of increase in antibody titer is higher in IgG2a, and the increase in the expression of Th1 cytokines is also higher. These results also suggested that oral administration TCDD skews the Th1/Th2 balance to Th1.

Dioxins act suppressively on the adaptive immune response [9,16,21–23]. However, the immune response was enhanced in this study. This difference is likely because most dioxins are administered at 1 μg/kg or higher as a single dose administration, and antigens are intraperitoneally administered using an adjuvant such as alum. In addition, it has been reported that TCDD disrupts oral immune tolerance [24]. Our data suggested that ingestion with TCDD disrupted oral tolerance and increased antibody production. We found that TCDD disrupts the barrier function of epithelial cells and promotes the penetration of substances (unpublished data). Therefore, we consider that the

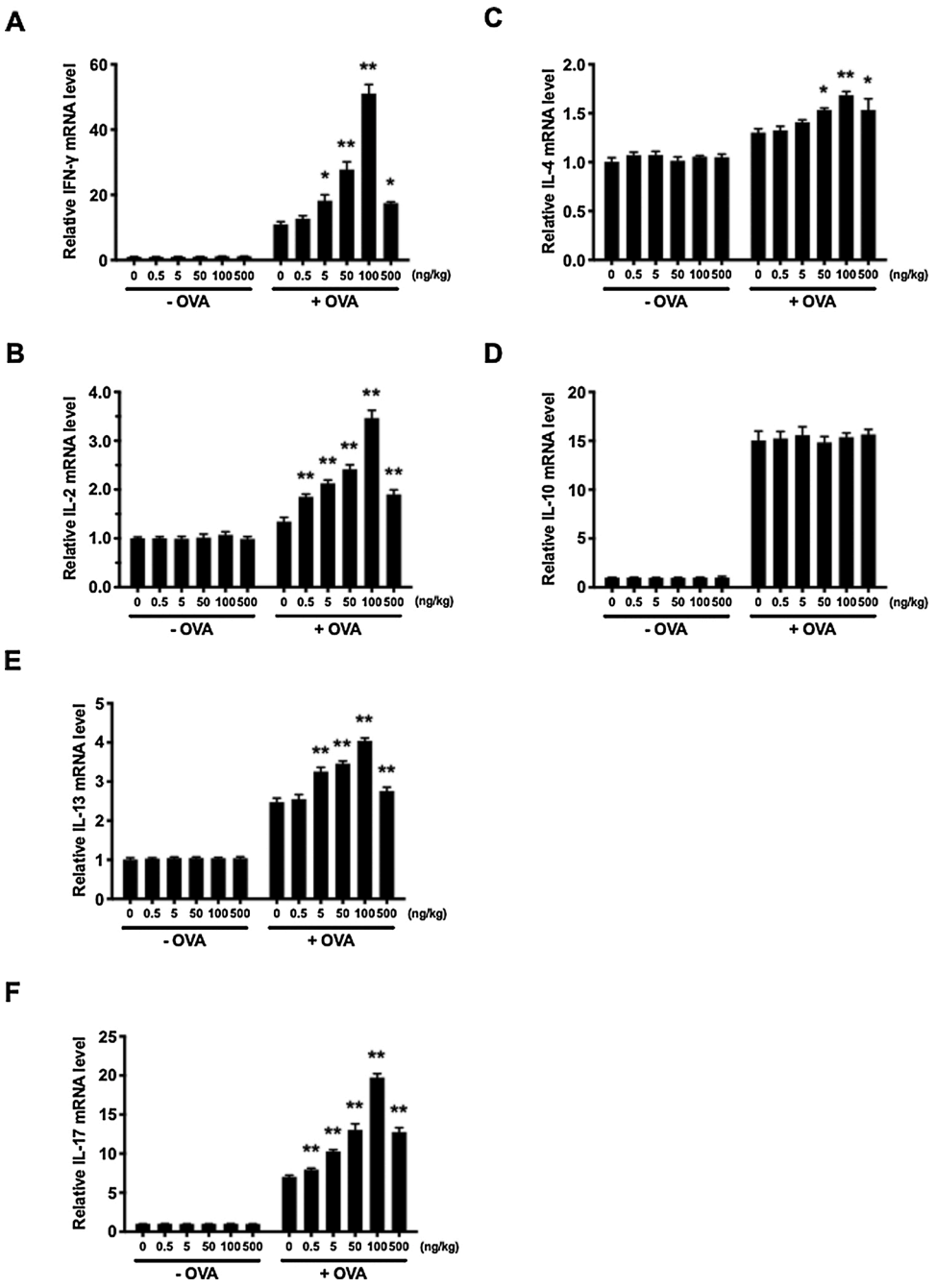

Fig. 2. Induction of cytokine mRNA in splenocytes.
Mice were orally administrated TCDD and OVA daily for 10 weeks. At week 10, splenocytes isolated from the immunized mice were stimulated with vehicle or OVA (1 mg/mL) for 2 h, and cytokine (IFN-γ, IL-2, IL-4, IL-10, IL-13, and IL-17) mRNA levels were measured by qPCR. The data are shown as means ± SE (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with vehicle.

presence of TCDD allows for the excessive permeation of the antigen, which enhances antibody production.

Different animal species have different susceptibilities to dioxins. In particular, C57BL/6 mice are approximately five times more sensitive than DBA/2 mice [25]. It is possible that the results of this study were due to using BALB/c instead of C57BL/6 mice, which are generally used in dioxin research. However, no significant difference was observed between the two mouse strains except for 50 ng/kg when evaluated by ethoxyresorufin *O*-deethylase (EROD), which is a general method for evaluating the toxicity of dioxins (Supplemental Fig. 1). Although there is a statistical significant at 50 ng/kg, the activities of both mice can be sufficiently confirmed, and the difference is only about 1.1 times. This suggests that the results of this study were not specific to the animal strain.

In Japan, the tolerable daily intake (TDI) of dioxins is 4 pg-TEQ/kg b. w./day [26]. If a 60-kg adult consumes the TDI, it is equivalent to 240 pg-TEQ/adult/day. However, estimating the average mouse body weight as 20 g is equivalent to 100 pg/mouse/day at 5 ng/kg/day. No significant increase in antibody production was obtained at 5 ng/kg/day, but it showed an increasing tendency. It should accumulate in the body by daily intake of dioxins. Thus, we think that increased antibody production by dioxins could occur sufficiently through daily diet.

In conclusion, we evaluated the effects of subclinical TCDD exposure against the immunological system, including antibody production. Our results suggest that low doses of TCDD might show adjuvanticity, high dose of that might show immunosuppression effects. Additional studies are required to elucidate the specific effects and molecular mechanisms of low-dose, long-term dioxin exposure.

Author statement

Hideki KAKUTANI: Methodology, Writing - Original Draft **Tomohiro YUZURIHA:** Validation, Formal analysis **Teruyuki NAKAO:** Visualization **Souichi OHTA:** Supervision.

Conflict of interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.12.011.

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