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Original Article

Breakdown of Mucosal Immunity in Gut by 2,3,7,8-Tetraclorodibenzo-*p*-dioxin (TCDD)

Hirokazu KINOSHITA[1,2], Jun ABE[1], Kenji AKADEGAWA[3], Hideaki YURINO[1], Tetsuya UCHIDA[4], Shigaku IKEDA[2], Kouji MATSUSHIMA[1] and Sho ISHIKAWA[1]

[1]*Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Japan*
[2]*Department of Dermatology, Juntendo University School of Medicine, Japan*
[3]*Department of Respirology, Graduate School of Medicine, Chiba University, Japan*
[4]*Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, National Institute of Infectious Diseases, Tokyo, Japan*

Abstract

Objectives: Mucosal immunity plays a pivotal role for body defense against infection and allergy. The aim of this study was to clarify the effects of 2,3,7,8-tetraclorodibenzo-*p*-dioxin (TCDD) on mucosal immunity in the gut.

Methods: Fecal IgA level and oral tolerance induction were examined in TCDD-treated mice. Flow cytometric and histological analyses were also performed.

Results: Single oral administration of low dose 2,3,7,8-TCDD resulted in a marked decrease in IgA secretion in the gut without any effects on the cellular components of gut-associated lymphoid tissues (GALT) including Peyer's patches (PPs) and mesenteric lymph nodes (LNs). Decreased IgA secretion by TCDD was not observed in aryl hydrocarbon receptor (AhR)-deficient mice. Flow cytometric analysis revealed that IgA^+ B cells in PPs and the mesenteric LNs remained unchanged in the TCDD-treated mice. An immunofluorescence study also demonstrated that a significant number of cytoplasmic IgA^+ cells were present in the lamina propria of the gut in the TCDD-treated mice. Furthermore, oral tolerance induction by ovalbumin (OVA) was impaired in the TCDD-treated mice and OVA-specific T cell proliferation occurred in the peripheral lymphoid tissues including the spleen and LNs.

Conclusions: These results suggest that a relatively low dose of TCDD impairs mucosal immunity in the gut and induces systemic sensitization by oral antigens.

Key words: TCDD, mucosal immunity, IgA, oral tolerance, allergy

Introduction

TCDD has been reported to exert a variety of adverse effects on immune responses including antibody production and cytotoxic T lymphocyte (CTL) generation (1–5). Exposure to TCDD also results in decreased resistance to several infectious agents (6–8). However, the immunological effects of dioxins on mucosal immunity in the gut have not been intensively examined to date, despite the fact that most dioxin exposures occur in the digestive tract. Intestinal mucosal immunity is characterized by massive IgA secretion into the gut lumen and the induction of oral tolerance against large amounts and different types of dietary antigens. Both intestinal IgA and oral tolerance play a pivotal role in body defense to protect against pathogens and to prevent systemic allergic sensitization by oral antigens (9–11). We previously demonstrated that mucosal immunity in the gut was impaired in a (New Zealand Black × New Zealand White) F1 hybrid mouse strain (BWF1), a murine model for systemic lupus erythematosus (SLE) (12, 13). Aged BWF1 mice developing lupus nephritis showed defective IgA secretion in the gut and increased susceptibility to bacterial infection. Oral tolerance was also impaired and orally administered antigens induced systemic allergic sensitization in the

Abbreviations: OVA, ovalbumin; PPs, Peyer's patches; LNs, lymph nodes; CTL, cytotoxic T lymphocytes; BWF1, (New Zealand Black × New Zealnad White) F1 hybrid; SLE, systemic lupus erythematosus; HRP, horse radish peroxidase; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanate; CFA, complete Freund's adjuvant; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; TGF, transforming growth factor; GVH, graft versus host reaction.

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Reprint requests to: Dr. Sho ISHIKAWA
Department of Molecular Preventive Medicine, School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
TEL: +81(3)5841-3677, FAX: +81(3)5841-3393
E-mail: yamasho@m.u-tokyo.ac.jp

respiratory tract in these mice. On the other hand, it is well recognized that the incidence of allergic diseases has been increasing over the past several decades in developed countries and that environmental factors are more involved in "increasing the incidence of disease" than genetic factors (14–16). These environmental factors include increased degree of air pollution, and increased amounts of dust mites, dietary antigens, and environmental chemicals, among others (17, 18). We hypothesize that environmental chemicals that disrupt mucosal immunity in the gut would result in allergic sensitization by oral antigens and could be a critical environmental factor in the increase in the incidence of allergic diseases.

We found that the administration of low-dose TCDD resulted in defective IgA secretion in the gut in an AhR-dependent manner and in the breakdown of oral tolerance. Antigen-specific systemic sensitization was established in TCDD-treated mice and the pathological significance of impaired mucosal immunity by TCDD in allergic diseases is discussed.

Materials and Methods

Mice

Specific pathogen-free C57BL/6J mice, originally obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained under SPF conditions in our animal facility at The University of Tokyo. Female mice aged 6–8 wk were intragastrally administrated with TCDD (Daiichikagaku Co., Tokyo, Japan) in corn oil. TCDD at 1 μg/kg was administrated to mice except in the dose-response experiment. All experiments except dose-response curve in Fig. 1 All animal experiments complied with the standards contained in the guidelines for the use and care of laboratory animals in the University of Tokyo. AhR deficient mice were originally generated from a C57BL/6 background by Schmidt et al. (19) and kindly provided by Dr. C. Tohyama (The University of Tokyo) with a permission of Dr. R. E. Peterson (University of Wisconsin). The AhR genotype was determined by RT-PCR analysis using specific primers as described by Benedict et al. (20). Five mice 6–8 wk old were used for each experimental group. No growth retardation was observed in the adult mouse colony.

Cell preparation

Mice were sacrificed under ether anesthesia to prepare cell suspensions from lymphoid organs. Peritoneal B1 cells were purified using MACS® magnetic beads (Miltenyi Biotech.) from whole peritoneal cells. Briefly, T cells, macrophages and B2 cells were depleted by incubating with a biotinylated mAb cocktail (anti-Thy1.2, anti-F4/80 plus anti-CD23 mAbs) followed by incubation with streptavidin-conjugated magnetic beads. Splenic B2 cells and $CD4^+$ T cells were also isolated using MACS beads conjugated with anti-mouse B220 or anti-mouse CD4 mAb. Cell purity was more than 90% throughout the experiments.

ELISA for fecal IgA

One hundred milligrams of fecal pellets was placed into 1.5 ml microcentrifuge tubes: 1 ml (10 volumes, w/v) of PBS was added and the tubes were incubated at room temperature for 15 min. The fecal samples were vortexed, left to settle for 15 min, revortexed until all materials were suspended, then centrifuged at 12,000 rpm for 10 min. The supernatant was removed and stored at −80°C or immediately tested using ELISA kit for IgA (Bethyl Laboratories, Montgomery, TX). Microtiter plates were coated with goat anti-mouse IgA affinity purified antibody and incubated for 60 min. Plates were washed with PBST (PBS containing 0.05% Tween 20) and each well was blocked with 200 μl of 50 mM Tris (pH 8.0) containing 0.15 M NaCl and 1% BSA for 30 min. After washing with PBST, 100 μl each of the test samples and standards was added per well and incubated for 60 min. Horse radish peroxidase (HRP)-labeled goat anti-mouse IgA-Fc specific Ab was added to each well and incubated for 60 min. Color was developed with a HRP substrate (3,3',5,5'-tetramethyl benzidine) for 30 min and read at 450 nm with using an Emax® precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

Flow cytometry

Fluorescein-isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5), anti-CD5 (53-7.3RRH) and anti-CD11b (M1/70); Phycoerythrin (PE)-conjugated anti-CD8α (53-6.7), anti-CD11c (HL-3), and anti-B220 (RA3-6B2); and allophycocyanin (APC)-conjugated anti-B220 (RA3-6B2) mAbs were purchased from PharMingen (San Diego, CA). Lymphoid cells were stained with 1) FITC-conjugated anti-CD4, PE-conjugated anti-CD8 and APC-conjugated anti-B220 mAbs, or 2) FITC-conjugated anti-CD5 and PE-conjugated anti-B220 mAbs, or 3) FITC-conjugated anti-CD11b and PE-conjugated anti-CD11c mAbs and analyzed on an Epics Elite® cell sorter (Coulter Electronics, Hialeah, FL).

Immunofluorescence study

PPs, mesenteric LNs, and spleen tissue samples were embedded in Tissue-Teck® embedding compound (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Six-micron-thick cryostat sections were incubated with FITC-conjugated anti-mouse IgA Ab, PE-conjugated anti-B220 Ab, and APC-conjugated anti-collagen type IV Ab (Biomedical Technology, Inc.) and observed under a fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Induction of oral tolerance

The induction of systemic unresponsiveness to OVA (Sigma Chemical Co., St. Louis, MO) was performed as described previously (7). Briefly, mice were given 25 mg of OVA in 250 μl of PBS by gastric intubation on Day 0. Control mice received PBS. On Day 7 and 21, mice were immunized and challenged subcutaneously (s.c.) with 100 μg of OVA in 100 μl of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI). The OVA specific Ab level in the serum was measured 7 days after the second s.c. immunization.

OVA-specific serum Abs by ELISA

Anti-OVA Ab titers in serum samples were determined by ELISA. Briefly, ELISA plates (Corning Incorporated Life

Sciences, Acton, MA) were coated overnight at 4°C with 1 mg/ml OVA in PBS. Blocking was performed with 200 µl of 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Serial dilutions of serum in 1% BSA/PBS were prepared and 100 µl was added per well in duplicate. Following incubation at 37°C for 4 h, HRP-labeled goat anti-mouse IgG-Fc specific Abs (Bethyl Laboratories) were added and incubated overnight at 4°C. Color was developed with 1.1 mM 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical Co.) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H_2O_2.

OVA induced cell proliferation assay

Twenty-five milligrams of OVA in 250 µl of PBS was administered intragastrally three times in the next week after the TCDD treatment (Day 0). Two weeks after the last administration, the spleen and lymph nodes (axillar, pulmonary, mesenteric, renal, and inguinal) were removed aseptically. Single-cell suspensions were obtained using fine-mesh screens (Cell Strainer, Becton Dickinson, Franklin Lakes, NJ). Cells (4×10^5) were cultured in the presence of OVA or keyhole limpet hemocyanin (KLH) (200 µg/ml) for 5 days at 37°C in a 5% CO_2 atmosphere. Each well was pulsed with ^{3}H-thymidine (1 µCi/ml) for the last 18 h of the culture. Cells were then harvested onto a glass filter and radioactivity was determined using a liquid scintillation counter.

Results

Decreased IgA secretion in the gut by oral administration of TCDD

To investigate the immunological effects of TCDD on the intestinal mucosa, C57BL/6 mice were intragastrally administered various doses of TCDD and the fecal IgA level was determined by ELISA. The fecal IgA level was dose-dependently decreased in mice treated with TCDD (Fig. 1A). Note that relatively low doses of TCDD (0.1 µg/kg and 1.0 µg/kg) significantly inhibited IgA secretion into the gut lumen. The fecal IgA level returned to the normal level by 4 weeks after the administration of 1 µg/kg TCDD (Fig. 1B). To determice whether the inhibitory effect of TCDD on IgA secretion in the gut is mediated by AhR, which is a specific receptor for TCDD, the fecal IgA level in AhR-deficient mice administered with a TCDD (1 µg/kg) was examined. The inhibitory effect of TCDD on IgA secretion in the gut was totally abrogated in AhR-deficient mice with a C57BL/6 background, whereas heterozygous littermates and C57BL/6 mice showed a marked decrease in IgA secretion in the gut (Fig. 2).

Flow cytometric analysis

FACS analysis revealed that there was no significant change in the cellular compartments ($CD4^+$, $CD8^+$, $B220^+$, and $CD11b^+11c^+$ cells) in PPs, and in the thymus, spleen, and LNs in mice treated with 1 µg/kg TCDD (Fig. 3A–E). The percentages of $CD11b^+CD11c^+$ cells, a cell surface phenotype for myeloid type dendritic cells, in PPs, and in the mesenteric LNs and spleen were 0.25±0.07, 0.25±0.07, and 0.6±0.01 in the TCDD treated mice and 0.3±0.01, 0.2±0.01, and 0.55±0.07 in the control mice, respectively. The absolute number of each subset

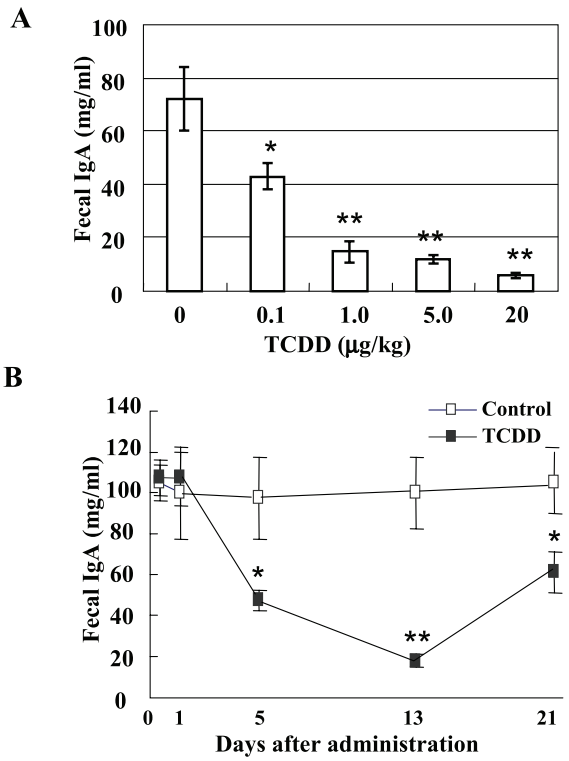

Fig. 1 TCDD suppression of IgA secretion in gut. A. Dose-dependent IgA suppression by TCDD. C57BL/6 mice (n=5) were intragastrally administered TCDD (0.1, 1.0, 5, 20 µg/kg) and the fecal IgA level was determined by ELISA 1 wk after TCDD treatment. Monoclonal IgA Ab was used as a control. The mean concentrations±SD are presented in each graph. A representative result from four independent experiments is presented. B. Kinetic study of IgA suppression by TCDD. Mice (n=5) were given 1 µg/kg TCDD (filled squares, ■) or corn oil (open squares, □). Feces were collected on days 1, 5, 13, and 21 and the fecal IgA level was determined by ELISA on the same plate on the same day.

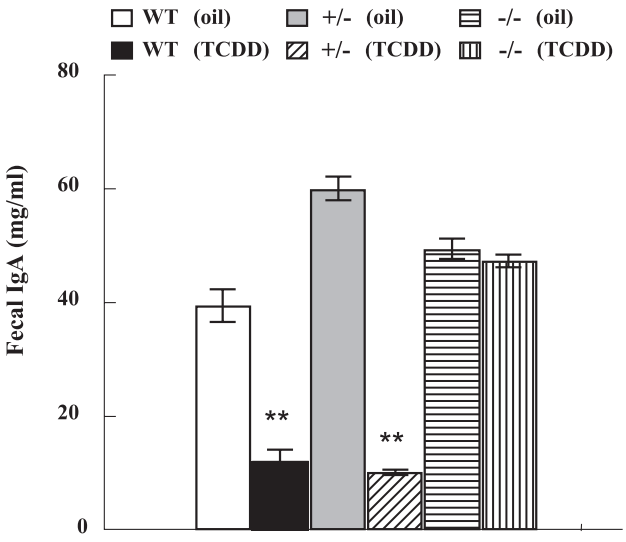

Fig. 2 AhR-dependent suppression of IgA secretion in TCDD-treated mice. TCDD (1 µg/kg) or corn oil was given to C57BL/6 (WT), AhR heterozygous (+/−), and AhR-deficient (−/−) mice and the fecal IgA level was determined by ELSA 1 wk after TCDD treatment (n=5). A representative result from two independent experiments is presented. Statistical analysis was performed using the Student's *t*-test. ** $p<0.001$.

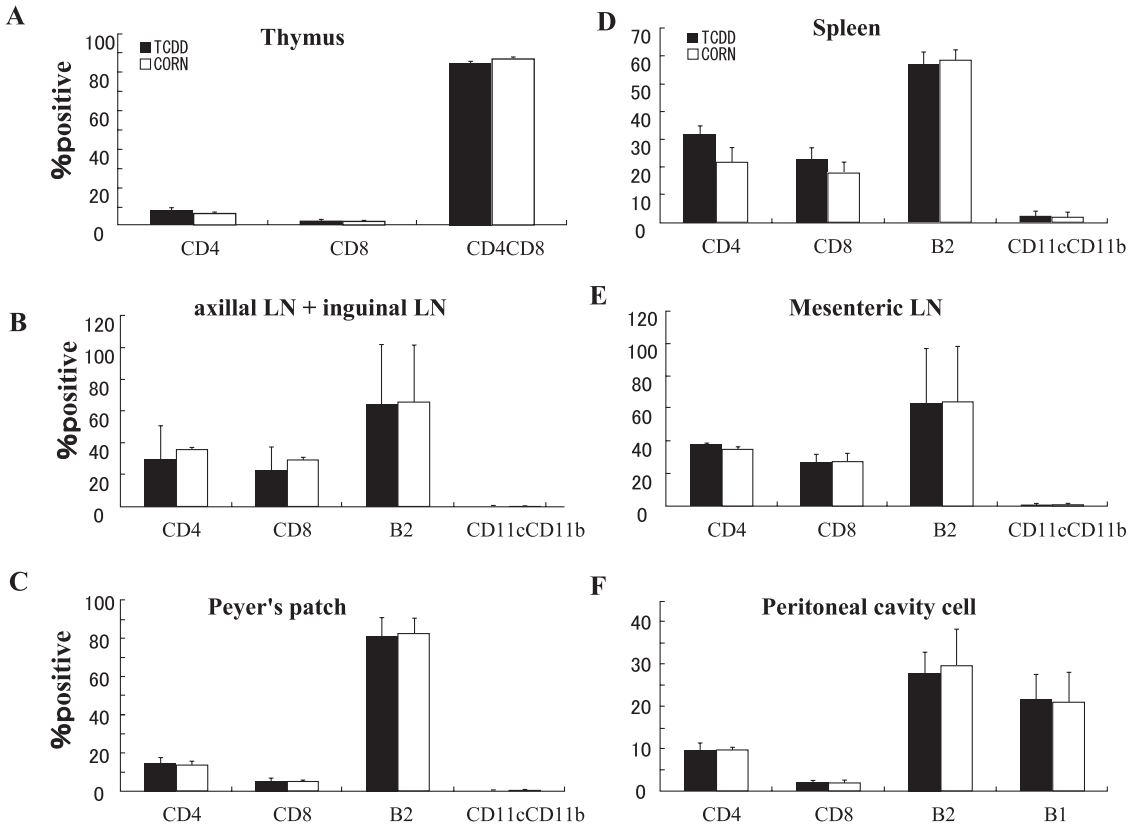

Fig. 3 FACS analysis on lymphoid tissues. Cells from the thymus, spleen, and LNs were stained with a mixture of FITC anti-CD4, PE anti-CD8, and APC-anti-B220 Abs or FITC anti-CD11b and PE anti-CD11c Abs and analyzed using a flow cytometer. Peritoneal cells were stained with FITC anti-CD5 and PE-B220 antibodies to discriminate B1 ($CD5^+B220^{int}$) and B2 ($CD5^-B220^{high}$) cells. The mean percentages±SD are presented in each graph (n=4). A representative result from three independent experiments is presented.

was not changed either (data not shown). Mean fluorescence intensities for CD4, CD8 and B220 molecules also remained unchanged in TCDD-treated mice (data not shown). Note that the percentage of peritoneal B1 ($CD5^+B220^+$) cells remained unchanged in the TCDD-treated mice (Fig. 3F).

No histological change and presence of IgA^+ cells in TCDD-treated mice

Hematoxylin-Eosin (H-E) staining showed that the intestinal mucosa remained unchanged in the TCDD-treated mice (Fig. 4A). To determine whether defective IgA secretion in the gut can be attributed to a decreased percentage of IgA^+ cells in the gut-associated lymphoid tissues (GALT), FACS analysis and an immunofluorescence study were performed. FACS analysis on the B cells in PPs and the mesenteric LNs revealed that the frequency of IgA^+ cells remained unchanged in the TCDD-treated mice (Fig. 4B). The immunofluorescence study also demonstrated that a significant number of cytoplasmic IgA^+ cells (plasma cells) were present in the lamina propria of the gut in the TCDD-treated mice as well as in the control mice (Fig. 4C).

Effect of TCDD on oral tolerance

Oral tolerance is historically and originally described as the antigen specific inhibition of antibody production by oral preadministration of protein antigen. As shown in Fig. 5A, OVA-specific IgG production was suppressed in mice that had been orally administered OVA before systemic immunization, demonstrating that oral tolerance was induced in the control mice. The oral administration of KLH did not inhibit OVA-specific IgG production (data not shown). In contrast, the suppression of IgG production was partially abrogated in the TCDD-treated mice, suggesting a breakdown of oral tolerance. To examine the effect of TCDD treatment on antigen uptake, mice were administered 1 mg of Alexa488-labeled OVA one week after TCDD treatment. Alexa488-labeled OVA was incorporated into the subepithelial dome (SED) of PPs, a major site for antigen uptake in the gut, similarly in the TCDD-treated and control mice (Fig. 5B).

Consistent with the impaired oral tolerance in the TCDD-treated-mice, lymphocytes in PPs, and in the axillar, inguinal, and cervical LNs, and the spleen of TCDD-treated mice antigen-specifically proliferated in vitro in the presence of OVA, whereas KLH stimulation did not induce cell proliferation (Fig. 6).

Discussion

We have demonstrated in this study that the oral administration of a relatively low dose of TCDD results in a marked decrease in IgA secretion in the gut. However, FACS analysis revealed that the number of IgA^+ B cells was not decreased in the mesenteric LNs and PPs in the TCDD-treated mice. An immunofluorescence study also showed that a significant

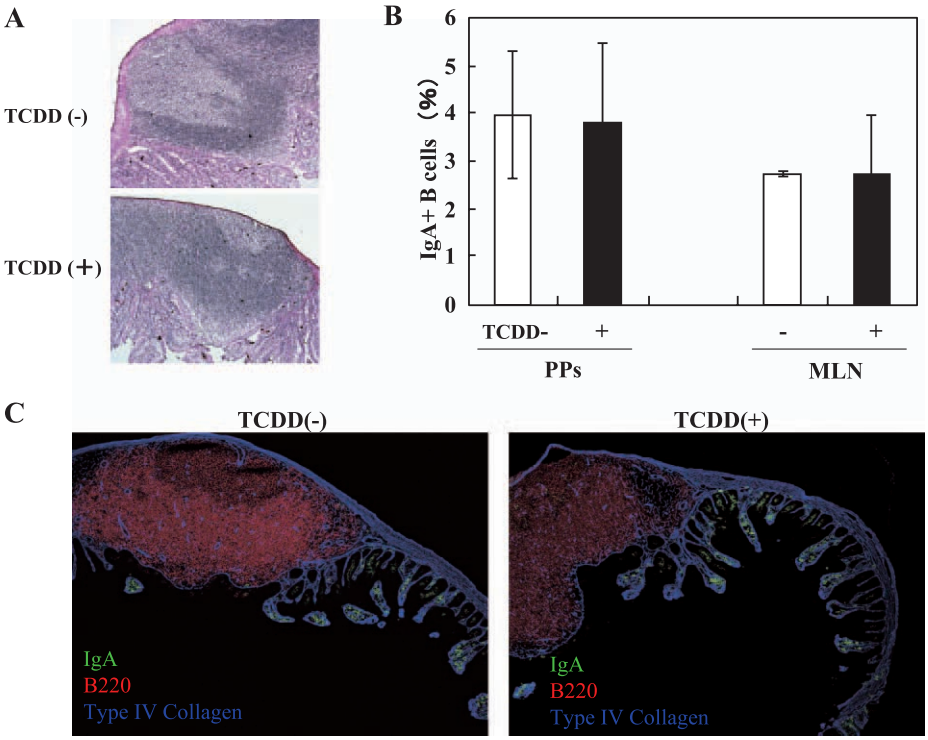

Fig. 4 Histological change in intestinal mucosa and presence of IgA$^+$ cells in TCDD-treated (1 μg/kg) mice. A. Hematoxylin-Eosin (H-E) staining of intestinal mucosa of TCDD-treated mice showed no pathological changes such as inflammation or necrosis (×100). B. Cells prepared from PPs or mesenteric LNs were stained with FITC-anti-IgA, PE-anti-B220, and APC-anti-CD19 Abs. The percentages of IgA$^+$B220$^+$ cells among CD19$^+$ cells are presented (n=3). C. Cryosections of intestinal mucosa were stained with FITC anti-IgA, PE-anti-B220, and APC anti-collagen Type IV Abs and examined under a confocal laser scanning microscope (×100). Many IgA$^+$ cells (green) were observed in the intestinal lamina propria in the TCDD-treated mice as well as in the control mice.

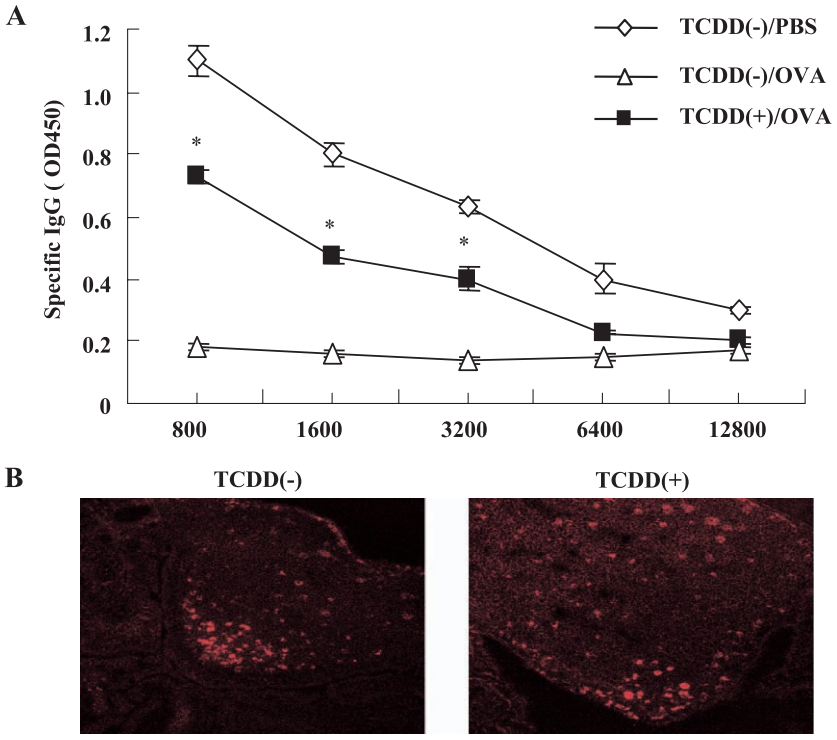

Fig. 5 Breakdown of oral tolerance and systemic sensitization by oral antigens and intact antigen uptake in TCDD-treated (1 μg/kg) mice. A. Mice were treated with TCDD (filled squares, ■) or corn oil (open triangles, △) on Day 0 and orally administered 25 mg of OVA on Days 7 and 14. Then, mice were immunized s.c. with 100 μg of OVA in CFA on Days 21 and 28 (n=4). Mice immunized with OVA plus CFA were used as positive controls (open diamonds, ◇). The serum concentration of OVA-specific IgG on Day 35 was determined by ELISA. The results are expressed as mean±SD. Representative data from three experiments are presented. * $p<0.001$. B. Alexa488-labeled OVA was administrated intragastrally and cryostat sections prepared 2 h after administration were analyzed under a fluorescence microscope (×100). The labeled OVA localized mainly in the subepithelial dome (SED) of PPs both in the TCDD-treated and control mice.

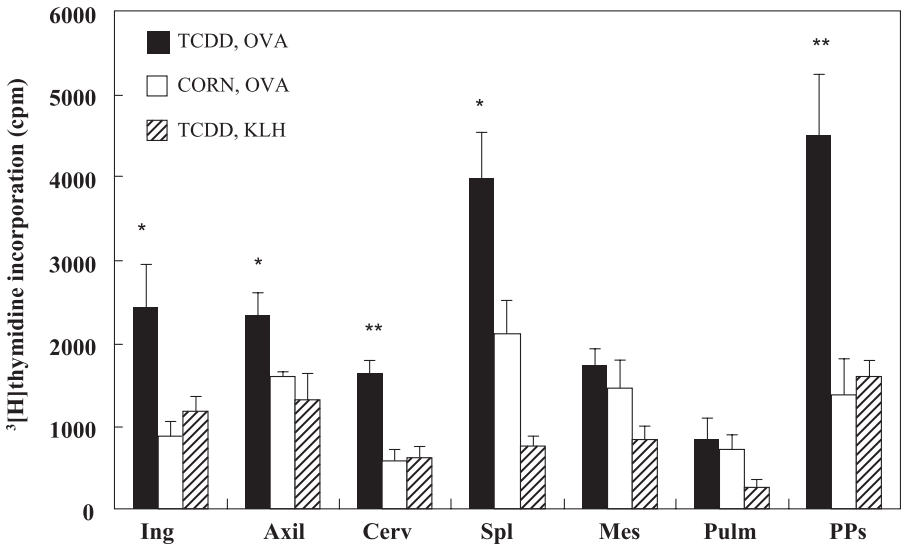

Fig. 6 Systemic sensitization with orally administered OVA by TCDD pretreatment (1 μg/kg). Mice were treated with TCDD on Day 0 and then mice were intragastrally given 25 mg of OVA twice on Day 7, 10 and 14. On Day 28, single-cell suspensions were obtained from the spleen (Spl), and inguinal (Ing), axillar (Axil), mesenteric (Mes), and pulmonary (Pulm) lymph nodes, and PPs and stimulated in vitro with OVA or KLH (200 μg/ml) at 37°C for 5 days. Cell proliferation was measured by ^{3}H-thymidine incorporation as described in Materials and Methods. Results are presented as means±SD (n=4). Representative data from three experiments are presented. * $p<0.05$, ** $p<0.01$.

number of IgA^+ cells were present in the lamina propria of the gut in the TCDD-treated and control mice. Cellular subsets of immunocompetent cells in lymphoid tissues including PPs, and the spleen, thymus, and peripheral LNs, remained unchanged.

It has recently been reported that the constitutively AhR causes selective loss of peritoneal B1a ($CD5^+B220^{low}IgM^{high}$) cells (21), suggesting that B1 cells are sensitive cellular targets for TCDD. B1 cells are a specialized cell population distinguished from conventional B cells (B2 cells) by their origin, cell surface phenotype, unique tissue distribution and capacity for self renewal; these cells have also been considered to be involved in autoantibody production in the development of autoimmune diseases (22). It is also believed that approximately half of the IgA^+ cells in the intestinal lamina propria are derived from B1 cells and B1 cells play a pivotal role in innate mucosal immunity in the gut (23, 24). In this study, however, the number and frequency of B1 cells in the peritoneal cavity were not decreased in the TCDD-treated mice. The expression of constitutively active AhR during ontogeny may result in different effects on B1 cells from those observed in our study. The mechanisms for defective IgA secretion in the gut remain to be elucidated. It is possible that TCDD affects the synthesis of secretory component or the secretion machinery of intestinal epithelial cells.

The breakdown of oral tolerance by TCDD is another interesting finding in this study. Oral tolerance is historically and originally described as the antigen-specific inhibition of systemic IgG production by oral preadministration of protein antigens (10, 11). Many studies have been performed using an experimental protocol similar to that used in this study to demonstrate the presence or absence of oral tolerance. However, the precise mechanism for oral tolerance still remains to be clarified (25). It was previously reported that regulatory T cells producing transforming growth factor (TGF)-β and/or IL-10 were induced in PPs by the oral administration of protein antigens (26, 27). However, the classical idea for the pivotal role of PPs in oral tolerance has been challenged by several studies demonstrating that oral tolerance could be induced independently of PPs (28, 29). It was also demonstrated that the spleen plays an important role in oral tolerance (30, 31). These results are in agreement with the idea that mesenteric LNs and the spleen are critical lymphoid organs functioning as induction sites for oral tolerance although they do not exclude the physiological role of PPs. Accumulating evidence also suggests that dendritic cells (DCs) play a pivotal role in oral tolerance (32–34). However, the frequency of $CD11c^+$ cells remained unchanged in PPs, and the mesenteric LNs and spleen before and after the TCDD treatment. Funatake et al. (35) have recently demonstrated that TCDD generates $CD25^+CD4$ T cells with a regulatory function in a graft versus host (GVH) reaction. However, FACS analysis showed only a marginal increase in the frequency of $CD25^+CD4$ T cells in PPs, and the mesenteric LNs and spleen (6.3±0.28, 12.3±0.28, and 8.95±0.21 in the TCDD-treated mice and 4.25±0.35, 11.4±0.21, and 7.6±0.28 in the control mice, respectively). The route of antigen trafficking may be another factor affecting the immune response in the gut as indicated by our recent work on a murine model for SLE (13). However, alexa488-labeled OVA was incorporated into the subepithelial dome (SED), a major site for antigen uptake, similarly in the TCDD-treated and control mice. Functional analysis on DCs with different localizations is under way to elucidate the mechanism involved in oral tolerance disruption by TCDD.

As a result of the breakdown of oral tolerance, T cells in PPs, and in the axillar inguinal, and cervical LNs were sensitized by the orally administered OVA. It is known that patients with atopic dermatitis show a high frequency of food allergy and that dietary allergens such as egg albumin often turn out to be the allergen in the skin of these patients, indicating the existence of immunological cross talk between

the intestinal mucosa and the skin. It is considered that microbial infection, excessive antibiotic administration, and the early onset of a weaning diet among others are the causes of systemic allergic sensitization to oral antigens in infants. Our findings suggest that TCDD may be a possible candidate for such disruptors of mucosal immunity. Although 1 μg/kg TCDD is far more than the human adult daily intake, breast-fed newborn infants take in 15–20 times more TCDD than the tolerable daily intake (TDI). Furthermore, the immature mucosal immune system of newborn infants may be much more sensitive to TCDD exposure. The effects of TCDD on mucosal immunity in the gut in newborn infants will be further clarified in the near future.

Collectively, we have demonstrated that a relatively low dose of TCDD results in the breakdown of intestinal mucosal immunity and systemic sensitization by oral antigens in mice. The immunological health effects of environmental chemicals such as dioxins should be assessed on the basis of mucosal immunity in the gut. This may also provide a new insight for understanding environmental factors responsible for the increased incidence of allergic diseases in recent decades.

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