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Original Articles

Gut colonization by *Candida albicans* aggravates inflammation in the gut and extra-gut tissues in mice

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We examined whether *Candida albicans* gut colonization aggravates immune diseases in mice. Chronic and latent *C. albicans* gut colonization was established by the intragastric inoculation of *C. albicans* in mice fed as part of a purified diet. Allergic diarrhea was induced by repetitive intragastric administration of ovalbumin in sensitized BALB/c mice. Contact hypersensitivity was evaluated by measuring ear swelling after topical application of 2, 4-dinitrofluorobenzene in NC/Nga mice. Arthritis was induced by intradermal injection of bovine type-II collagen emulsified with complete Freund's adjuvant in DBA/1J mice. *C. albicans* gut colonization increased the incidence of allergic diarrhea, which was accompanied by gut hyperpermeability, as well as increased infiltration of inflammatory cells in the colon. Contact hypersensitivity was also exacerbated by *C. albicans* gut colonization, as demonstrated by increased swelling, myeloperoxidase activity, and proinflammatory cytokines in ear auricles. Furthermore, *C. albicans* gut colonization promoted limb joint inflammation in collagen-induced arthritis, in an animal model of rheumatoid arthritis. These findings suggest that *C. albicans* gut colonization in mice aggravates inflammation in allergic and autoimmune diseases, not only in the gut but also in the extra-gut tissues and underscores the necessity of investigating the pathogenic role of *C. albicans* gut colonization in immune diseases in humans.

Keywords *Candida albicans*, allergic diarrhea, contact hypersensitivity, collagen-induced arthritis, mice

Introduction

Candida albicans is part of the indigenous microbial flora of the human gastrointestinal tract [1]. Since Truss described in 1985 that tissue injury induced by *C. albicans* was accompanied by mental and neurological manifestations [2], it was postulated that the excessive colonization by *C. albicans* in the gut may be responsible for wide ranging unspecific chronic symptoms such as fatigue, diarrhea, food intolerance, arthritis, and skin problems which was called '*Candida* hypersensitivity syndrome' [2–5]. Mechanistically, it was believed that immune dysfunction, coupled with multiple allergies, contributed to this list of clinical complaints. For example, it has been hypothesized that excessive colonization by *C. albicans* in the gastrointestinal mucosa may be aggravating factors in atopic dermatitis [6–8]. However, in a recent review, Goldman and Huffnagle described that the data are not definitive and that more research is needed to understand any cause-and-effect relationships between *C. albicans* gut colonization and a variety of hypersensitivity diseases including allergy [9].

In animal studies, Noverr *et al*. reported that *C. albicans* gut colonization promotes allergic airway inflammation in response to mold spore (*Aspergillus fumigatus*) or ovalbumin (OVA) in antibiotic-treated immunocompetent mice [10,11]. Because these mice repeatedly received intranasal

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antigen administration without systemic immunization, the authors mentioned that *C. albicans* gut colonization coupled with antibiotic treatment can disrupt normal airway immune tolerance in mice [11]. In addition, we have developed a model of sustained *C. albicans* gut colonization by a single intragastric inoculation of *C. albicans* in healthy adult mice without administration of antibiotics or immunosuppressants [12]. Although these mice appear healthy despite lifelong *C. albicans* gut colonization under immunocompetent conditions, disseminated infection by *C. albicans* in visceral organs including spleen, kidneys, liver, and lungs is induced upon treatment with immunosuppressive agents. Thus, these mice are useful as an animal model mimicking immunocompetent humans with chronic and latent gut colonization by *C. albicans*. Using this model, we demonstrated that serum antibody responses to repeated oral administration of OVA were enhanced by *C. albicans* gut colonization, suggesting that *C. albicans* gut colonization is likely to increase the risk for food allergy [13]. In the present study, we addressed whether *C. albicans* gut colonization aggravates allergic and autoimmune diseases in mice.

Materials and methods

Animals

The following study was approved by the Hokkaido University Animal Use Committee (approved no. 08-0139), and animals were maintained in accordance with the guidelines of Hokkaido University for the care and use of laboratory animals. Specific pathogen-free 5-week-old female BALB/c and NC/Nga mice and male DBA/1J mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were housed in a temperature-controlled (23 $\pm$ 2°C) room with a dark period from 20:00 to 08:00 and allowed free access to water and a purified diet prepared according to AIN-93G [14].

Inoculation and enumeration of C. albicans

C. albicans (JCM 1542) was maintained as previously described [12]. For inoculation, all mice were acclimatized to the purified diet for 2 weeks before being deprived of the diet for 16 h. Mice were then inoculated intragastrically with 0.2 ml of saline containing 1×10^7 cells of *C. albicans* (Candida [+]). Control mice were intragastrically administered 0.2 ml of the vehicle (Candida [−]). Fecal specimens were quantitatively cultured using a standard pour plate technique as previously described [12].

Allergic diarrhea

Allergic diarrhea was induced in BALB/c mice following the method of Kweon *et al*. [15]. Twelve mice were inoculated with *C. albicans* as described above, and another 12 mice were uninoculated. At 1 week after the inoculation, 6 mice in each group were immunized subcutaneously with 1 mg of OVA (grade V, Sigma, St. Louis, MO) in 100 µl of complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, MI). Another set of 6 mice in each group was injected subcutaneously with 100 µl of phosphate-buffered saline (PBS, unimmunized). Two weeks after the immunization, all mice started to receive 10 mg of OVA dissolved in 250 µl of saline by intragastic administration every other day. Diarrhea was assessed by visually monitoring mice for up to 2 h following intragastric challenge. Mice demonstrating liquid stool were recorded as diarrhea-positive animals. After the 10th challenge, mice were anesthetized by inhalation of diethyl ether, and whole blood was drawn from the carotid artery. Serum was separated from the blood samples and subjected to ELISA for measurement of OVA-specific antibody titers as described below. Following a laparotomy, the entire length of the colon was excised and, after flushing the luminal contents with 10 ml of ice-cold PBS, a 1-cm section of the proximal colon was embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and stored at −80°C for histological examination as discussed below. The remaining colon was subjected to the *in vitro* permeation experiment as described below.

Contact hypersensitivity

Contact hypersensitivity (CHS) to 2, 4-dinitrofluorobenzene (DNFB) was induced in NC/Nga mice following the method of Nagai *et al*. [16]. Six mice were inoculated with *C. albicans* as described above, and another 6 mice were uninoculated. At 3 weeks after the inoculation, 25 µl of 0.15% (v/v) DNFB (Tokyo Kasei, Tokyo, Japan) in acetone/olive oil (4:1, v/v) and the vehicle were applied to each side of the right and left ear auricles of all mice, respectively, twice at 7-day intervals. Ear thickness was measured with a digital engineer's micrometer (Mitsutoyo, Kawasaki, Japan) before and 24, 48, and 72 h after each application of DNFB. Ear thickness measurements were performed by an investigator who was blinded to the mouse treatments. DNFB-specific ear swelling was calculated according to the following equation:

> Net swelling = (right ear thickness – left ear thickness) at each time point – (right ear thickness – left ear thickness) at 0 h.

After the last measurement of ear thickness, mice were anesthetized by inhalation of diethyl ether, and whole blood was drawn from the carotid artery. Serum was separated from the blood samples and subjected to ELISA for

measurement of dinitrophenol (DNP)-specific antibody titers as described below. Ear auricles were excised, and a portion of each tissue was embedded in OCT compound and stored at −80°C for histological examination as described below. The remaining tissue samples were subjected to myeloperoxidase (MPO) activity measurement and mRNA expression analysis as discussed below.

Collagen-induced arthritis

Bovine type-II collagen (CII, Cosmo Bio, Tokyo, Japan) was dissolved in 0.05 M acetic acid to a concentration of 2 mg/ml by stirring overnight at 4°C and then emulsified with an equal volume of CFA. Six DBA/1J mice were inoculated with *C. albicans* as described above, and 12 mice were uninoculated. At 2 weeks after the inoculation, 150 μl of the emulsion (150 μg CII) was injected intradermally on the back of 6 Candida [+] and 6 Candida [−] mice. At 3 weeks after the primary immunization, mice were boosted intradermally with 150 μl of the emulsion (150 μg CII) at the base of the tail. Another set of 6 Candida [−] mice was injected with PBS (i.e., untreated). After the boosted injection, the clinical severity of arthritis (i.e., arthritis score) was assessed on a daily basis for visual appearance of each paw, and was quantified using the following index: 0, normal; 1, swelling of a single finger; 2, swelling of multiple fingers; 3, swelling of the entire paw. Each paw was graded, and one mouse could thus have a maximum score of 12. Additionally, the thickness of each paw was measured with a digital engineer's micrometer (Mitsutoyo) on day 0, 3, 6, 10, 17, and 21 after the boosted injection. Paw thickness measurements were performed by an investigator who was blinded to the mouse treatments. Blood samples were obtained from the tail vein at weekly intervals and subjected to ELISA for measurement of CII-specific antibody titers as described below. On the last day of the experiment (i.e., day 21), mice were anesthetized by inhalation of diethyl ether, and whole blood was drawn from the carotid artery. Serum was separated from the blood samples and subjected to measurement of β-glucan as described below. Each limb was excised, and the joint tissues were prepared by removing the skin and then separating the paw below the ankle joint. The tissue samples were subjected to MPO activity measurement and mRNA expression analysis as described below.

ELISA for serum antibodies

Serum antibodies specific to OVA, DNP, and CII were measured by ELISA as previously described [12]. For anti-OVA IgG, 96-well microtiter plates were coated with OVA, blocked, and incubated with serially diluted serum samples. Bound IgG was detected by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody (Zymed Laboratories, South San Francisco, CA). Anti-OVA IgE was captured with rat anti-mouse IgE monoclonal antibody (LO-ME-2, Zymed Laboratories)-coated microtiter plates and detected with digoxigenin-conjugated OVA followed by HRP-conjugated sheep anti-digoxigenin Fab fragments (Roche Diagnostics, Tokyo, Japan). Anti-DNP IgG1 and IgG2a were captured with DNP-bovine serum albumin (Calbiochem, San Diego, CA)-coated microtiter plates and detected with HRP-conjugated rat anti-mouse IgG1 (clone LO-MG1-2, Zymed Laboratories) or rat anti-mouse IgG2a (clone LO-MG2a-3, Zymed Laboratories). Anti-CII IgG was captured with CII-coated microtiter plates and detected with HRP-conjugated goat anti-mouse IgG polyclonal antibody (Zymed Laboratories). Plates were developed at room temperature after the addition of *o*-phenylenediamine (Sigma) and hydrogen peroxide. Pre-immunized serum was used as a negative control. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference for determination of the titer in the test sera. Antibody titers were expressed as the reciprocal of the last dilution yielding an extinction value higher than the reference value.

Histology

Cryostat sections (5 μm) were prepared and stained with hematoxylin and eosin (H&E). Toluidine blue staining was also performed to identify mast cells in the colon tissue sections. In the colon tissue sections, the number of eosinophils and mast cells was counted using a high-power field in a section from each specimen. All cell counts were performed by a single observer who was blinded to the mouse treatments.

Permeation of HRP in the colon in vitro

As described by Enomoto *et al*. [17], translocation of HRP for 40 min was measured in isolated segments of colon. In brief, 5-cm segments of colon were everted, filled with 200 μl of Tris-HCl buffer (125 mM NaCl, 10 mM fructose, 30 mM Tris, pH 7.5), and ligated at both ends. The filled gut segments were incubated in Tris-HCl buffer containing 40 μg/ml HRP (Sigma) at 37°C. After 40 min, gut sacs were removed, and the contents of each sac were collected. HRP activity in the contents of each sac was determined spectrophotometrically from the rate of oxidation of 3, 3′, 5, 5′-tetramethylbenzidine (TMB, Sigma).

MPO activity

Tissue samples were homogenized in a 50 mM phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethyl

ammonium bromide (Sigma). The homogenates were then subjected to three freeze/thaw cycles. After the centrifugation at 13,000 *g* for 30 min at 4°C, the supernatants were subjected to MPO activity measurement as described by Bánvölgyi *et al*. [18]. In brief, the samples were added to a 50 mM phosphate buffer (pH 6.0) supplemented with hydrogen peroxide and TMB, and optical density readings were then taken for 15 min at 620 nm. The reaction rate (Δ OD/time) was derived from an initial slope of the curve. A calibration curve was then produced, with the rate of reaction plotted against the standard samples of the human MPO (Sigma). Aliquots of tissue homogenate were subjected to mRNA expression analysis as described below.

mRNA expression analysis

Total RNA was isolated from tissue homogenates using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After digestion of genomic DNA with RQ1 RNase-free DNase (Promega, Madison, WI), approximately 10 ng of total RNA was annealed with Oligo (dT) $_{12\text{-}18}$ primer (Invitrogen) at 70°C for 10 min, and 1st strand cDNA was then synthesized using M-MLV reverse transcriptase (Invitrogen), followed by RNA digestion with DNase-free RNase H (Invitrogen). Real-time quantitative PCR (RT-qPCR) was performed using Thermal Cycler Dice TP800 (Takara, Ohtsu, Japan). Primer sequences for interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were identical to Giulietti *et al*. [19]. Amplification was carried out in a 25-μl reaction volume containing 12.5 μl 1 × SYBR Premix Ex *Taq* (Takara), 200 nM of each primer and 1 μl of template cDNA. The reaction condition was: 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s, with dissociation curve at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Relative gene expression levels for each sample were normalized to the levels for GAPDH.

β-glucan measurement

Serum samples (5 μl) were pretreated with 20 μl of a solution containing 0.6 M KCl and 0.125 M KOH for 10 min at 37°C and assayed with the Glucatell reagent kit (Associates of Cape Cod, East Falmouth, MA) in a kinetic, chromogenic format for 35 min at 37°C following the manufacturer's instruction. Optical densities at 405 nm were read by using a microplate reader (Synergy Mx, BioTek Instruments, Winooski, VT). The concentration of β-glucan in each sample was calculated by using a calibration curve with standard solutions of 3.125 to 50 pg/ml.

Statistics

Results are presented as mean ± SEM. Unpaired *t*-test or Tukey-Kramer's test following one-way or two-way analysis of variance was used to compare mean values. The χ^2 test was used to compare the frequencies of diarrhea. StatView for Macintosh (version 5.0, SAS institute Inc., Cary, NC) was used for the analyses.

Results

Effect of C. albicans *gut colonization on allergic diarrhea in BALB/c mice*

After intragastric inoculation, a high fecal recovery of *C. albicans* was observed in all Candida [+] mice throughout the experimental period (Fig. 1A). Enumeration of *C. albicans* in the gut tissues by quantitative culture after euthanasia of animals revealed that colonization occurred in the stomach, jejunum, ileum and colon in all Candida [+] mice (data not shown). We detected no *C. albicans* in the feces and tissues of Candida [−] mice. The present study did not examine the colonization of other fungal species in the gut of mice.

Diarrhea was observed after two oral administrations of OVA to the systemically immunized Candida [+] mice (Fig. 1B). As described by Kweon *et al*. [15], diarrhea was observed within 30 min after OVA challenge and diminished within 2 h, suggesting that acute gut allergic responses had occurred in these mice. In the immunized Candida [−] mice, diarrhea was first observed after six OVA challenges. The incidence of diarrhea in both groups roughly continued to increase by the end of the experiment. However, Candida [+] mice showed a higher incidence of diarrhea throughout the induction period. Repeated OVA challenge in mice without systemic immunization did not induce any change in the gross appearance of feces (data not shown).

H&E staining of colon sections showed that eosinophils infiltrated the epithelium and crypt region of the colon in immunized mice (Fig. 1D, upper panels). Eosinophils were rarely observed in the colon of unimmunized mice (Fig. 1E). In immunized mice, the number of infiltrated eosinophils was significantly higher in Candida [+]mice than in Candida [−] mice, and was significantly higher in mice with diarrhea than in mice without diarrhea (51 ± 13 *vs.* 225 ± 24 cells/mm^2, $P < 0.0001$). Additionally, toluidine blue staining showed the infiltration of mast cells in the colon (Fig. 1D, lower panels). In both immunized and unimmunized mice, the number of infiltrated mast cells was significantly higher in Candida [+] mice than in Candida [−] mice (Fig. 1E). In both Candida [+] and Candida [−] mice, those with immunization had a significantly higher number of mast cells in the colon as compared to mice without immunization. In immunized mice, the

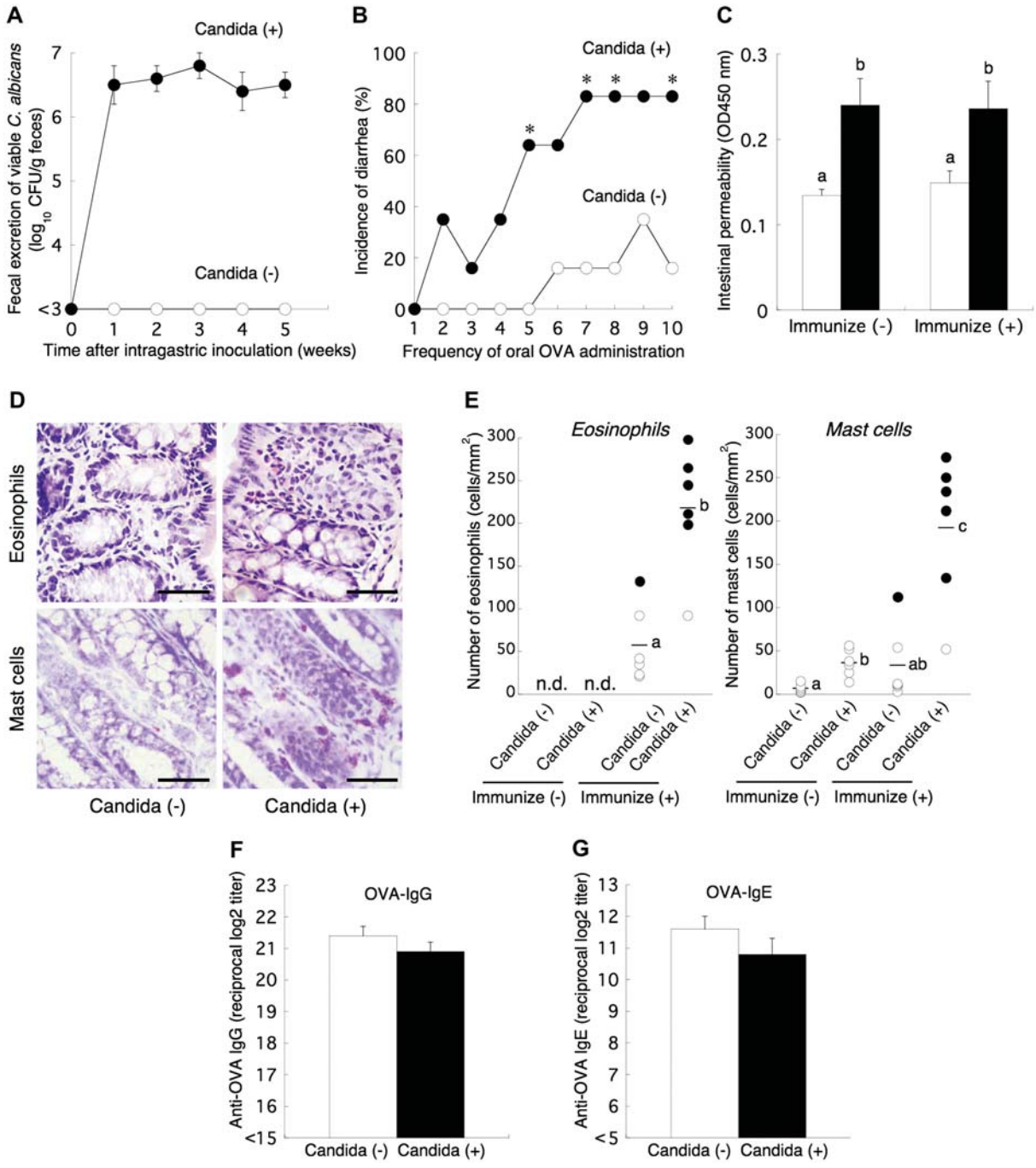

Fig. 1 Effect of *Candida albicans* gut colonization on OVA-induced allergic diarrhea in BALB/c mice. (A) Temporal changes in the recovery of viable organisms from the feces in mice with (Candida [+], closed circle) and without (Candida [−], open circle) intragastric *C. albicans* inoculation. Values are represented as mean ± SEM of six mice per group. (B) Temporal changes in diarrhea incidence in Candida [+] and Candida [−] mice (closed and open circles, respectively). Values with an asterisk are significantly different ($P < 0.05$) from values of Candida [−] mice at each time point as estimated by χ^2 test. (C) *In vitro* gut permeability in Candida [+] and Candida [−] mice (closed and open columns, respectively). Gut permeability was estimated using translocation of HRP in isolated segments of colon. Values are represented as mean ± SEM of six mice per group. Values with unlike letters are significantly different ($P < 0.05$) as estimated by Tukey-Kramer's test. (D) Representative H&E staining and toluidine blue staining for eosinophils and mast cells, respectively, in colon sections of mice after 10th OVA challenge. Bars represent 50 μm. (E) Numbers of infiltrated eosinophils and mast cells in colon sections of mice. Each circle represents the value of individual mice, and closed and open circles represent mice with and without diarrhea, respectively. Horizontal bars represent mean values. Values with unlike letters are significantly different ($P < 0.05$) as estimated by Tukey-Kramer's test. n.d., not detected. (F and G) IgG and IgE titers specific to OVA in Candida [+] and Candida [−] mice (closed and open columns, respectively). Values are represented as mean ± SEM of six mice per group.

number of mast cells was significantly higher in mice with diarrhea than in mice without diarrhea (24 ± 9 *vs.* 202 ± 27 cells/mm^2, $P < 0.0001$).

Gut permeability was estimated by measuring translocation of HRP in isolated segments of colon after completing 10-times administration of OVA (Fig. 1C). In both immunized and unimmunized mice, the translocation of HRP was significantly higher in Candida [+] mice than in Candida [−] mice. Systemic immunization had no effect on the translocation of HRP.

Figures 1F and 1G shows serum antibody titers specific to OVA after the last administration. There was no significant difference between Candida [+] and Candida [−] mice for both IgG and IgE. Mice without systemic immunization showed undetectable levels of both IgG and IgE antibodies specific to OVA (data not shown).

Effect of C. albicans *gut colonization on contact hypersensitivity in NC/Nga mice*

Intragastric inoculation of *C. albicans* in NC/Nga mice led to a high fecal recovery of *C. albicans* in all mice throughout the experimental period (data not shown). The first application of DNFB induced no ear swelling in all mice (Fig. 2B), but did begin to be detected 24 h after the second challenge and tended to decrease thereafter until 72 h. Ear swelling was significantly higher in Candida [+] mice than in Candida [-] mice from 24–72 h after the second challenge. Histological examination shows moderate cell infiltration and severe edema in the dermis of challenged ear auricle sections (Fig. 2A). In the challenged ear auricle sections, thickness of dermis was significantly higher in Candida [+] mice than in Candida [−] mice (268 ± 32 *vs.* 405 ± 56 μm, $P < 0.05$). In addition, there was a significant correlation between the last measurement of ear swelling as measured by hand-operated thickness gauge and dermis thickness as measured in the histological section ($r = 0.891$, $P < 0.01$).

Neutrophil infiltration in the inflamed ear auricles on day 10 after the first ear challenge was indirectly quantified by MPO activity in the tissue homogenates. In the vehicle-treated left ear auricles, no detectable levels of MPO activity were observed (data not shown). MPO activity in the right ear auricles was significantly higher in Candida [+] mice than in Candida [−] mice (Fig. 2C). Additionally, expression of proinflammatory cytokine genes in ear auricles at day 10 after the first ear challenge was quantified by RT-qPCR. Figure 2D shows the fold induction of IL-1β, IL-6, and TNF-α genes in DNFB-treated ear auricles, relative to the values of vehicle-treated ear auricles, which were taken as 1. These proinflammatory cytokines were upregulated by intradermal injection of CII. The expression levels of these genes were significantly higher in Candida [+] mice than in Candida [−] mice.

There was no detectable hapten-specific antibody in the sera of mice without DNFB treatment (data not shown). On day 10 after the first application of DNFB, all the mice produced detectable levels of hapten-specific IgG1 and IgG2a antibodies (Figs. 2E and 2F, respectively). There was no significant difference between Candida [+] and Candida [−] mice relative to both IgG1 and IgG2a.

Effect of C. albicans *gut colonization on collagen-induced arthritis in DBA/1J mice*

DBA/1J mice inoculated with *C. albicans* showed high *C. albicans* fecal recovery throughout the experimental period (data not shown). Figure 3A illustrates the gross appearance of mouse limbs. As compared to untreated mice, swelling of forepaws and hindpaws was obvious in CII-challenged Candida [+] mice. However, in Candida [−] mice, slight swelling was observed in hindpaws, whereas swelling of forepaws was not evident. Quantitatively, the arthritis score began to increase on day 5 after boosted immunization with CII (Fig. 3B). The score was significantly higher in Candida [+] mice than in Candida [−] mice from day 13 to day 21. Additionally, paw thickness began to increase on day 10 after boosted immunization (Fig. 3C). In left forepaws, the thickness was significantly higher in Candida [+] mice than in Candida [−] mice from day 10 to day 21. Right forepaws also tended to be thicker in Candida [+] mice than in Candida [−] mice. The thickness of hindpaws tended to be higher in Candida [+] than in Candida [-] mice, and the difference was statistically significant in both right and left hindpaws on day 21.

Untreated mice had no detectable levels of MPO activity in joint tissue homogenates (data not shown). MPO activity in both forepaws and hindpaws tended to be higher in Candida [+] mice than in Candida [−] mice (Fig. 3D). Expression of proinflammatory cytokine genes in joint tissues was quantified for pooled samples in each group. Figure 3E shows the fold induction of IL-1β, IL-6, and TNF-α genes in challenged mice, relative to the values of untreated mice, which were taken as 1. These proinflammatory cytokines, particularly IL-6, were upregulated by intradermal injection of CII. The increase in the expression of these genes was more evident in Candida [+] mice than in Candida [−] mice.

There was no detectable CII-specific antibody in the sera of mice without immunization (data not shown). In immunized mice, CII-specific IgG antibody titers continued to increase by the end of the experiment in both groups of Candida mice (Fig. 3F). There was no significant difference between Candida [+] and Candida [−] mice at any time point after boosted injection of CII.

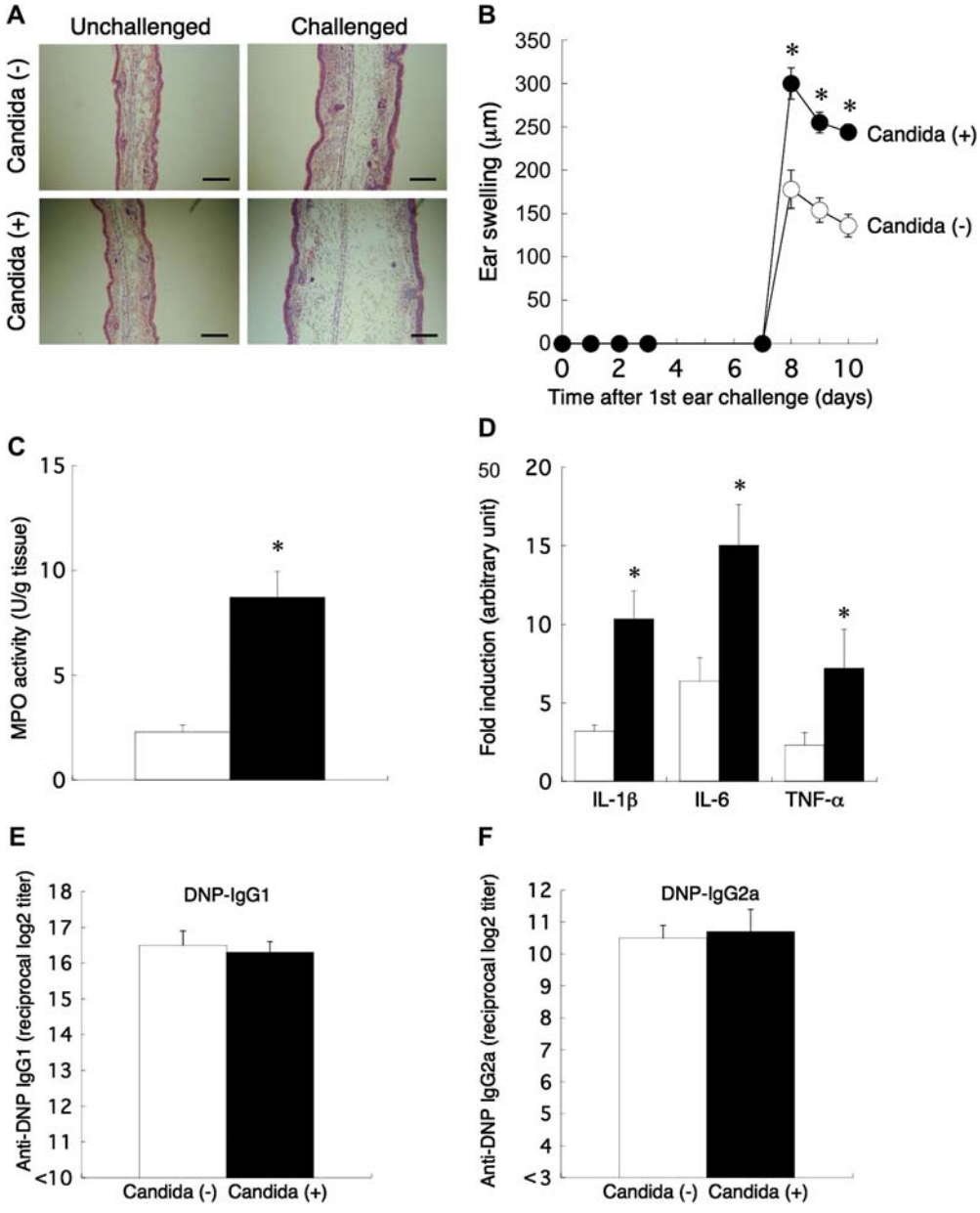

Fig. 2 Effect of *Candida albicans* gut colonization on DNFB-induced CHS in NC/Nga mice. (A) Representative H&E staining of ear auricles on day 10 after the first ear challenge. Bars represent 50 μm. (B) Temporal changes in ear swelling of Candida [+] and Candida [−] mice (closed and open circles, respectively). Values are represented as mean ± SEM of six mice per group. Values with an asterisk are significantly different ($P < 0.05$) from values of Candida [−] mice at each time point as estimated by unpaired *t*-test. (C) MPO activity in ear auricles of Candida [+] and Candida [−] mice (closed and open columns, respectively) on day 10 after the first ear challenge. Values are represented as mean ± SEM of six mice per group. Values with an asterisk are significantly different ($P < 0.05$) from values in Candida [−] mice as estimated by unpaired *t*-test. (D) Expression of IL-1β, IL-6, and TNF-α genes in ear auricles of Candida [+] and Candida [−] mice (closed and open columns, respectively) on day 10 after the first ear challenge as estimated by RT-qPCR. Values represent the fold induction of each gene in challenged mice, relative to the values of untreated mice, which were taken as 1. Values are represented as mean ± SEM of six mice per group. Values with an asterisk are significantly different ($P < 0.05$) from values in Candida [−] mice as estimated by unpaired *t*-test. (E and F) IgG1 and IgG2a titers specific to DNP in Candida [+] and Candida [−] mice (closed and open columns, respectively) on day 10 after the first ear challenge. Values are represented as mean ± SEM of six mice per group.

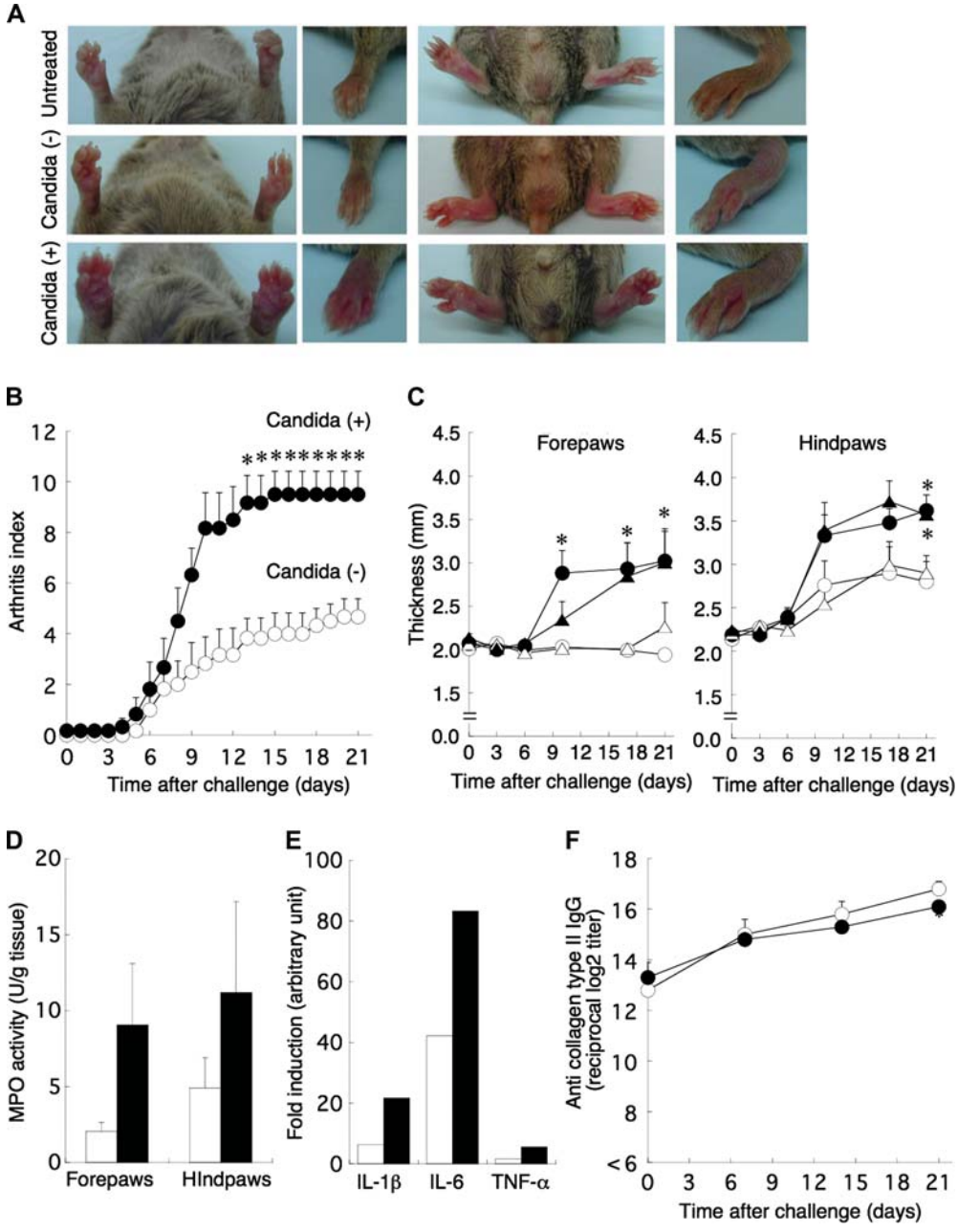

Fig. 3 Effect of *Candia albicans* gut colonization on CII-induced arthritis in DBA/1J mice. (A) Gross appearance of forepaws and hindpaws of Candida [+], Candida [−], and untreated mice on day 21 after boosted immunization. (B) Temporal changes in arthritis scores in Candida [+] and Candida [−] mice (closed and open symbols, respectively). Circles and triangles represent left and right paws, respectively. Values are represented as mean ± SEM of six mice per group. Values with an asterisk are significantly different ($P < 0.05$) from values in Candida [−] mice at each time point as estimated by unpaired *t*-test. (C) Temporal changes in paw thickness in Candida [+] and Candida [−] mice (closed and open circles, respectively). Values are represented as mean ± SEM of six mice per group. Values with an asterisk are significantly different ($P < 0.05$) from values in Candida [−] mice at each time point as estimated by unpaired *t*-test. (D) MPO activity in limb joint tissue homogenates of Candida [+] and Candida [−] mice (closed and open columns, respectively). Values are represented as mean ± SEM of six mice per group. (E) Expression of IL-1β, IL-6, and TNF-α genes in limb joint tissues of Candida [+] and Candida [−] mice (closed and open columns, respectively) as estimated by RT-qPCR. Values show the measurements of pooled samples in each group and represent the fold induction of each gene in challenged mice, relative to the values of untreated mice, which were taken as 1. (F) IgG titers specific to CII in Candida [+] and Candida [−] mice (closed and open circles, respectively). Values are represented as mean ± SEM of six mice per group.

Serum β-glucan concentrations were the same between Candida [−] mice and Candida [+] mice (16.4 ± 0.7 vs. 15.6 ± 1.9 pg/ml, respectively).

Discussion

The present study demonstrated that chronic and latent gut colonization by *C. albicans* promoted acute allergic diarrhea and CHS-induced ear swelling in immunocompetent mice. The results suggest that *C. albicans* gut colonization aggravates not only IgE-mediated immediate-type allergy but also T cell-mediated delayed-type hypersensitivity. In addition, the inflammatory responses are exacerbated not only by *C. albicans* colonization within the gut tissue but also in the extra-gut tissues. Furthermore, *C. albicans* gut colonization aggravated CII-induced arthritis, an animal model of rheumatoid arthritis, implying a contribution to arthritic deterioration in autoimmune disease. To our knowledge, the present study is the first describing the aggravation of allergic and autoimmune diseases by *C. albicans* gut colonization in immunocompetent mice.

Mice with allergic diarrhea showed the infiltration of eosinophils and mast cells in the colon, which is consistent with the report of Kweon *et al*. [15]. Exacerbation of allergic diarrhea by *C. albicans* gut colonization was the result of an increased infiltration of these inflammatory cells. Additionally, hapten-induced CHS mice and CII-induced arthritic mice showed an increase in MPO activity, a marker of neutrophil infiltration in inflamed tissues, and the activity was further increased by *C. albicans* gut colonization. Furthermore, gene expression of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α in inflamed tissues was upregulated by *C. albicans* gut colonization. Despite these enhanced inflammatory responses, *C. albicans* gut colonization exerted no changes in serum antigen-specific antibodies in three experimental models. Therefore, it is likely that *C. albicans* gut colonization modulates local inflammatory responses but not systemic humoral immune responses.

Our present study showed an increased protein permeation in the colon of *C. albicans*-colonized mice, as evidenced by *in vitro* translocation of HRP in isolated segments of the colon. Because we previously demonstrated that *C. albicans* gut colonization enhanced *in vivo* gut permeation of intragastrically administered proteins (that is, HRP and OVA) in BALB/c mice [13], it appears likely that translocation of OVA in isolated segments of the colon is also promoted by *C. albicans* gut colonization. In our present investigation, increased HRP translocation in the colon was observed not only in immunized mice but also in unimmunized mice, even though the mice were not affected by diarrhea. The results suggest that in mice colonized by *C. albicans*, higher gut permeability is not a consequence, but rather one of the causes of an increased incidence of allergic diarrhea. Our previous study also suggested that mast cells are responsible for increased gut permeability in *C. albicans*-colonized mice. In this study, mast cell infiltration in the colon was promoted by *C. albicans* colonization in unimmunized mice. Thus, it is likely that the infiltration of mast cells in the colon by *C. albicans* colonization contributes to an increased occurrence of allergic diarrhea by increasing antigen uptake in previously sensitized mice. According to Kweon *et al*., mast cells and eosinophils are recruited by Th2 cytokines produced by $CD4^+$ T cells infiltrated in the colon of diarrhea-induced mice [15]. Therefore, the increased uptake of antigen by *C. albicans* gut colonization may lead to an infiltration of antigen-specific CD4+ T cells, which in turn further recruit effector cells, such as mast cells and eosinophils, in the colon of diarrhea-induced mice. However, our preliminary experiment showed that *C. albicans* gut colonization did not alter IL-4 production in isolated mesenteric lymph node cells restimulated *in vitro* with OVA (1 mg/ml) in sensitized mice (0.87 ± 0.17 and 0.51 ± 0.33 ng/ml in mice with and without *C. albicans* gut colonization, respectively, Sonoyama *et al.* unpublished data), thus we need to examine whether *C. albicans* gut colonization promotes infiltration of antigen-specific $CD4^+$ T cells and production of Th2 cytokines in the colonic mucosa. Mast cells act as the main effector cells in the development of IgE-mediated allergic responses by releasing chemical mediators such as histamines, leukotrienes, and cytokines, which exert clinical symptoms, including diarrhea, and further recruit other immune and/or inflammatory cells [20]. Taken together, it is likely that mast cells act as the trigger and effector in promoting allergic diarrhea in *C. albicans*-colonized mice.

In hapten-induced CHS and CII-induced arthritis, *C. albicans* gut colonization aggravated the inflammatory responses in tissues distal to the gut. Because no infections are evident in the visceral organs in this *C. albicans* colonized mouse model [12], it is unlikely that invading viable fungal cells are responsible for the local inflammatory responses. In our mouse model of *C. albicans* gut colonization, serum IgG antibodies specific to the cell wall fraction of *C. albicans* become detectable after *C. albicans* gut colonization [12]. Regarding rheumatoid arthritis, gut microbiota have been widely proposed as a potential environmental factor associated with the etiology of this disease [21]. Indeed, the cell walls of several enteric bacterial species are arthritogenic in animal models, and patients with early rheumatoid arthritis were found to have a different gut microbiota from that of control patients [22]. In terms of fungi, Hida *et al*. reported that injection of CII with β-glucan isolated from *C. albicans* induces arthritis in DBA/1 mice [23], suggesting that β-glucan, a cell wall constituent of *C. albicans*, acts as an

adjuvant for CII-induced arthritis. Taken together, these findings raise the possibility that degradation products of fungal cells and/or secreted compounds might be absorbed by the gut, systemically circulate, and subsequently influence systemic immune responses and/or local inflammatory reactions. However, in CII-induced arthritis model of our present study, serum concentrations of β-glucan in mice with *C. albicans* gut colonization did not differ from mice without colonization. In addition, serum antibody titers specific to CII were the same between mice with and without *C. albicans* gut colonization. Our preliminary experiments showed that *C. albicans* gut colonization induced no overt symptoms of arthritis in DBA/1J mice when CII was injected without CFA (Miki *et al*. unpublished observation). Furthermore, we observed that serum antibody responses to repeated oral administration of OVA were enhanced by *C. albicans* gut colonization [13], but not by daily intragastric administration of heat-killed *C. albicans* (Hata *et al*. unpublished observation). Therefore, it appears unlikely that tissue inflammation and/or antibody responses are promoted by systemically circulating cell constituents such as β-glucan derived from *C. albicans* colonized in the gut. We are currently investigating whether intestinal epithelial cells and/or gut-associated lymphoid tissues exposed directly to *C. albicans* produce some circulating factors that promote tissue inflammation and antibody responses.

Taken together, the present findings suggest that *C. albicans* gut colonization aggravates inflammation in allergic and autoimmune diseases in mice, not only in the gut but also in tissues distal to the gut. However, the present findings are derived from animal experiments and not human studies, thus we propose the necessity of investigating the pathogenic role of *C. albicans* gut colonization in allergic and autoimmune diseases in humans. In addition, further studies are needed to figure out whether observed proinflammatory effects are specific to *C. albicans* and whether anti-fungal treatment ameliorates enhanced inflammation. Furthermore, we observed no significant relationships between fecal *C. albicans* excretions and inflammation parameters in the present study (data not shown), thus we need to clarify the relationship between gut colonization levels and extent of tissue inflammation. Nevertheless, the present study provides an experimental tool for studying the underlying molecular and cellular mechanisms for the proinflammatory action of *C. albicans* colonized in the gut.

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