BACKGROUND: Production of antibodies that are specific for allergens is an important pathological process in inflammatory allergic diseases. These contain the antibodies against antigens of *Candida albicans*, one of the normal microbial flora in an intestinal tract. We studied the effects of the prednisolone administration on the production of anti-*Candida* antibodies in the gastrointestinally *C. albicans*-colonized mice.

Methods and Materials: BALB/c mice, treated with antibacterial antibiotics to decontaminate indigenous intestinal bacterial flora, were inoculated intragastrically with *C. albicans*. The mice, in which *C. albicans* grows intestinally, were administered prednisolone to induce temporary immunosuppression. The *Candida* growth in their intestinal tract and their antibody response to *Candida* were examined.

Results: Antibiotic treatment allowed establishment of *C. albicans* gastrointestinal colonization, but did not cause subsequent systemic dissemination of *C. albicans* in all the animals. When these animals received an additional treatment with prednisolone, they showed a significantly higher population of *C. albicans* in their feces than those of animals treated with antibiotics alone, and the organisms were recovered even from their kidney. This systemic dissemination by *C. albicans* appeared to be temporal, because all the mice survived without any symptoms for more than 2 months. Examination of the serum titers of total immunoglobulin (Ig)E antibodies and specific IgE and IgG antibodies against *Candida* antigens demonstrated that titers of total IgE increased, partially by day 14 and clearly at day 27, in prednisolone-treated *Candida*-colonized mice. Without prednisolone treatment, an increment of the serum titer was scarcely observed. By day 27, corresponding to the increase of total IgE, the anti-*Candida* IgE and IgG titer increased in mice of the prednisolone-treated group.

Conclusion: Administration of prednisolone to *Candida*-colonized mice can induce production of the IgG, IgE antibodies against *Candida* antigens, perhaps through temporal systemic dissemination of *Candida* from the intestinal tract.

Key words: *Candida albicans*, Gut colonization, Immunoglobulin E antibody

Production of anti-*Candida* antibodies in mice with gut colonization of *Candida albicans*

Shigeru Tansho^{1,CA}, Shigeru Abe²,
Hiroko Ishibashi², Masayasu Mitsuya¹,
Kayoko Wada¹, Tatsuo Ikeda¹, Nobuo Suegara¹,
Osamu Koshio¹, Yasuo Ono¹ and
Hideyo Yamaguchi²

¹Department of Microbiology and Immunology, Teikyo University School of Medicine, 2-11-1 Kaga Itabashi-ku Tokyo 173-8605, Japan and ²Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan

^{CA}Corresponding Author

Tel: +81 3 3964 1211

Fax: +81 3 5375 5284

E-mail: stansho@med.teikyo-u.ac.jp

Introduction

Candida albicans is known to be one of the intestinal microbial flora of healthy persons.¹ To this microbe, adults elicit cellular and/or humoral immune responses postulated to have some pathogenetic relevance to such allergic diseases as atopic dermatitis (AD)^{2–7} and food allergy.⁸ Savolainen *et al.* reported that AD patients frequently have a high titer of anti-*C. albicans* antibodies in their sera.⁹ *Candida* organisms are colonized in the intestinal tracts in these patients and are harbored in the nasal

cavity and buccal capsule in a saprophytic manner at high frequency, and a high titer of immunoglobulin (Ig)E antibody reacting with *Candida* is frequently detectable.⁹ Moreover, several recent examples have shown that oral administration of antifungal agents to AD patients has displayed therapeutic efficacy with improvement of dermatitis.^{4,10,11}

To study the pathogenetic roles of *C. albicans* in these allergic diseases, it is important to analyze the induction process of anti-*Candida* IgE and/or IgG antibodies in *Candida*-colonized individuals. However, it is not yet known how specific *Candida* IgE

antibodies are produced in modern human life. We assumed that intestinal translocation of *Candida* organisms might provide immunogenic stimulation to produce specific IgE and/or IgG antibodies. We reported previously that application of antibiotics and anti-inflammatory corticosteroids induced an overgrowth of *C. albicans* in the intestinal tract of mice.¹² Here, we find that prednisolone treatment of mice intestinally colonized by *C. albicans* induced production of specific IgE and IgG antibodies, perhaps through systemic dissemination of this fungus from the gut.

Materials and methods

Preparation of *Candida* inoculate for challenge

C. albicans TIMM 0239^{12,13} was grown in Sabouraud dextrose broth in an L-tube. After growing at 37°C overnight, cells were harvested by centrifugation, washed three times with saline, and adjusted to a cell density appropriate for inoculation to mice.

Animals and inoculation

All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University. To produce intestinally *C. albicans*-colonized mice, we used a modified method of Uchida *et al.*¹³ Specific pathogen-free female BALB/c mice, 6–8 weeks old (Japan SLC, Inc., Shizuoka, Japan) were given potable water containing 1 mg/ml of ampicillin (Meiji Seika Co., Tokyo, Japan) and 0.2 mg/ml of kanamycin (Meiji Seika Co.) until the end of the experiments. Each mouse was challenged intragastrically with 1×10^6 cells of *C. albicans* at a volume of 0.1 ml using a gastric gavage. For immunosuppression, animals were subcutaneously given 100 mg/kg body weight of prednisolone (Mitaka Seiyaku, Tokyo, Japan) 7 and 9 days after the *Candida* challenge as described elsewhere.¹² Intestinal colonization of *C. albicans* was monitored by counts of viable *C. albicans* cells in their stools as follows. Every stool sample was homogenized in a volume of sterile saline and serial 10-fold dilutions with saline were made. One hundred microliters of each dilution was inoculated onto *Candida* GS agar (Tanabe Seiyaku Co. Ltd, Osaka, Japan) and the cultures were incubated at 37°C for 24 h, at which time quantitation of fungal colonies was performed. In some experiments, *Candida*-challenged mice were killed for microbial examination; kidneys were excised aseptically and homogenized in a glass tissue grinder with 1 ml of saline. Viable *Candida* cells were counted as already described, and the results were expressed as the mean

$\pm$ standard deviation value of colony-forming units (CFU) per kidneys of five mice in each group.

Enzyme-linked immunosorbent assay

The total IgE level was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using two kinds of rat anti-mouse IgE monoclonal antibody (mAb) (6HD5 and HMK12) according to the instructions of the manufacturer (Yamasa Shoyu Co., Ltd., Choshi, Japan).¹⁴ These mAbs recognized different epitopes of Fc fragments of murine IgE. Briefly, 96 wells of solid immunomicroplates (Nunc) were coated with 50 μ l of 6HD5 mAb (5 μ g/ml) and blocked with phosphate-buffered saline supplemented with 1% bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA). Collected samples or standard mouse IgE (SPE7; Seikagaku Kogyo, Tokyo, Japan) were added to the wells and incubated for 1 h at room temperature. Each well was washed with phosphate-buffered saline containing 0.05% Tween-20, and received 50 μ l of biotinylated HMK12 mAb (1 μ g/ml), and then all the plates were incubated for 1 h. After 50 μ l of peroxidase-conjugated avidin (1/2000; Dakopatts, Glostrup, Denmark) was added to each well, another 1 h incubation was carried out. Finally, the reaction products were visualized with 0.4 mg/ml of ortho-phenylenediamine (Sigma) and 0.012% H₂O₂. Thirty minutes after addition of the substrate, the reaction was stopped with 0.05 ml of 0.2 M H₂SO₄ and the absorbance at 490 nm wavelength was measured by an Immunoreader (NJ-2300; Nippon Intermed, Tokyo, Japan). In this assay the minimal detectable concentration of IgE was 40 ng/ml.

Specific IgE for *Candida* antigens was measured by an ELISA as described previously¹⁴ with slight modifications. A *Candida* antigen preparation was obtained from *C. albicans* cells by 37°C 2 h incubation in 0.05 M citrate buffer (pH 7) as described elsewhere.^{15–17} Immunomicroplates for ELISA (Nunc) were coated with the *Candida* antigen preparation (0.75 μ g protein/ml). Serum samples were incubated in the *Candida* antigen-coated microwells for 1 h. Then, binding of IgE antibodies with the *Candida* antigens was detected by rat anti-mouse IgE mAb (biotinylated HMK12 mAb) and peroxidase-conjugated avidin as already described.

Statistical analysis

Statistical difference in the survival rate was examined by Wilcoxon test. Other statistical analyses were examined by Student's *t*-test.

Results

Effects of treatments with antibiotics and/or prednisolone on intestinal colonization of *C. albicans* in mice

The effects of treatments to mice with two antibacterial agents, ampicillin and kanamycin, and/or prednisolone on the intestinal colonization of *C. albicans* were examined. BALB/c mice taking antibacterial agent-supplemented or unsupplemented drinking water were orally infected with *C. albicans*. As shown in Fig. 1, viable *Candida* cells were recovered from the feces of the *Candida*-inoculated mice from the day following oral inoculation (day 1). On this day the mean CFU number of *Candida* in the stools of mice given the supplemented drinking water was about 1×10^5 CFU/g of stool. The viable *Candida* population in the stools of mice given the unsupplemented drinking water rapidly decreased to a level of less than 1×10^4 CFU/g on day 3. On the other hand, antibiotic-treated mice maintained a high concentration of *C. albicans* of about 1×10^7 CFU/g for more than 2 weeks.

Figure 1 also shows that two subcutaneous administrations of prednisolone increased the CFU level about 10-fold in the antibiotic-treated mice. To check the systemic dissemination of *C. albicans* from intestinal colonization in these mice, their kidney homogenates of their kidney were cultured on *Candida*-GS-agar. As shown in Fig. 2, in mice treated with prednisolone at days 7 and 9, *Candida* cells were detected on day 11 and the number of CFU in

FIG. 1. Viable *Candida* cells in feces of *C. albicans*-infected mice treated with prednisolone. BALB/c mice were given potable water with (open symbols) and without antibiotic (closed symbols). Mice were infected with 10^6 CFU of *C. albicans* in their intestinal tracts, then were subcutaneously given 100 mg/kg of prednisolone (closed squares, open squares) or saline (closed circles, open circles) at 7 and 9 days after this infection, respectively. Feces counts were determined at time points thereafter. The results are expressed as a viable count obtained from groups of nine mice per data point.

FIG. 2. Viable *Candida* cells in the kidney of *C. albicans*-infected mice treated with or without prednisolone. BALB/c mice were infected with 10^6 CFU of *C. albicans* in their intestinal tracts, then were subcutaneously given 100 mg/kg of prednisolone (squares) and Saline (circles) at 7 and 9 days after this infection. *Candida* cell counts in kidney were determined at time points thereafter. The results are expressed as a viable count obtained from groups of nine mice per data point.

their kidney increased to 5×10^5 CFU/g on day 13, but the mice tested survived for more than 1 month. This indicates that the prednisolone treatment caused temporal systemic dissemination of *C. albicans* 2 weeks after the infection but did not result in *Candida* infection fatal to the hosts.

Antibody-formation of orally *Candida*-infected mice with or without prednisolone treatment

Total IgE concentration in sera of these *Candida*-infected mice at 2 and 4 weeks after *Candida* infection was measured. As shown in Fig. 3, in comparison with normal mice, a low but significant level of IgE (less than 100 ng/ml) could be detected in the sera of the antibiotic-treated and *Candida*-colonized mice at 2 weeks after the challenge. Prednisolone treatments of such mice clearly increased the serum level of IgE of the animals to about 120 ng/ml and 200–300 ng/ml at 2 and 4 weeks after the challenge, respectively.

In further experiments, the titers of IgE and IgG antibodies against *Candida* antigen in the sera of the *C. albicans*-colonized mice at 4 weeks after the challenge were examined. Figures 4 and 5 show that IgE and IgG, respectively, against *Candida* antigens increased in the serum of the animals treated with antibiotics and infected orally with *C. albicans* and twice administered with prednisolone. Without the treatment with both prednisolone and antibiotics, these infected mice would have had no sera showing a detectable level of antibodies against *Candida* antigens higher than those of control mice.

FIG. 3. Total serum IgE levels in *C. albicans*-infected mice treated with prednisolone. Serum levels were tested at 2 weeks (open circles) and 4 weeks (closed circles) after the *Candida* infections. Total IgE were measured using a specific ELISA as described in Materials and methods. Detection limits of total IgE were 40 ng/ml. * $p < 0.05$, when compared with the control (normal mouse serum).

These results indicated that prednisolone treatment caused a significant increase in specific *Candida* IgE and IgG antibodies in the sera of *Candida*-colonized mice.

Discussion

We have shown that prednisolone administration to mice with intestinally-colonized *C. albicans* induced IgG and IgE antibodies in their sera within 4 weeks after infection. As far as we know, this is the first report that administration of anti-inflammatory steroidal compounds causes production of anti-*Candida* antibodies in *Candida*-colonized animals. Antibody production augmented by prednisolone treatment is not curious, because immunosuppressive activity of prednisolone subcutaneously given in the depotsuspension form is known to disappear within 1 week after administration.

FIG. 4. Enhanced production of anti-*Candida* antibody (IgE) in sera of *C. albicans*-infected mice treated with prednisolone. Serum levels of antibodies were tested 4 weeks after the *Candida* infections. Specific IgE of anti-*Candida* antibody was measured using a specific ELISA as described in Materials and methods. Each value represents the mean $\pm$ standard error of five mice per group. * $p < 0.05$, when compared with the control (normal mouse serum).

The mechanism of the augmentation of antibody production by prednisolone remains to be clarified. We believe that prednisolone treatment must have a profound effect on the immunological condition of the mice, since the concentration of total IgE immunoglobulin in the sera clearly increased to 200–300 ng/ml in prednisolone-treated *Candida* infected mice. We can speculate that the augmentation of anti-*Candida* IgE production by prednisolone treatment may result from *in vivo* antigen stimulation accompanied by temporal systemic dissemination of *Candida* from the intestinal tract, since *Candida* dissemination to kidney was observed in the *Candida* infected prednisolone treated mice at 6 days after prednisolone treatment, as shown in Fig. 2. This speculation is supported by the finding that no augmented production of IgE or IgG against *Candida* antigens was observed in either antibiotic-treated or prednisolone-treated mice unless they had been orally inoculated with *C. albicans* (Figs 4 and 5)

The findings presented here may have some impact on the field of questions among clinical dermatologists of why steroidal anti-inflammatory drugs sometimes have a negative effect on the pathogenesis of AD. AD shows chronic symptoms with clinical manifestation of repeated aggravating itching and high concentration of IgE in sera. In AD patients dermal mast cells bind with IgE; when these

FIG. 5. Enhanced production of anti-*Candida* antibody (IgG) in sera of *C. albicans*-infected mice treated with prednisolone. Serum levels of antibodies were tested 4 weeks after the *Candida* infections. Specific IgG of anti-*Candida* antibody was measured using a specific ELISA as described in Materials and methods. Each value represents the mean $\pm$ standard error of five mice per group. * $p < 0.05$, when compared with the control (normal mouse serum).

IgE are bound with antigens, they release the chemical mediator histamine that induces pathological symptoms of edema and itching. Severe itching makes patients scratch their skin, perturbing/disturbing the epidermal organization of epidermal cells and destroying the barrier of the skin so that it is invaded by many allergens, worsening the dermatitis. These suggest that a high concentration of IgE in patient sera plays a critical role in the pathogenesis of AD. Our results therefore mean that a steroidal anti-inflammatory drug may have a severe pathogenic effect on AD patients because of the augmented production of IgE. However, at the present time, we cannot precisely explain the relationship among the administration of a steroid drug, intestinal translocation of *Candida* and pathogenesis of AD. To clarify this relation, we hope that our model in which anti-*Candida* IgE production can be induced by prednisolone treatment will be used as a tool to analyze the relationships between anti-inflammatory agents and IgE allergy with *Candida* infection.

References

1. Odds FC. *Candida and Candidosis*, Saunders WB, ed. London: Balliere Tindall; 1988.
2. Akiyama K, Shida T, Yasuega H, *et al.* Allergenicity of acid protease secreted by *Candida albicans*. *Allergy* 1996; **51**: 887–892.
3. Kimura M, Tsuruta S, Yoshida T. IFN-gamma plays a dominant role in upregulation of *Candida*-specific IgE synthesis in patients with atopic dermatitis. *Allergy. Clin Immunol* 2000; **122**: 195–199.
4. Kitamura K, Suga C, Onuma S. Efficacy of oral amphotericin B for refractory atopic dermatitis with specific IgE to *Candida albicans* and food allergens in sera. *Allergol Int* 1997; **46**: 125–133.
5. Nermes M, Savolainen J, Kalimo K, Lammintausta K, Viander M. Determination of IgE antibodies to *Candida albicans* mannan with nitrocellulose-RAST in patients with atopic diseases. *Clin Exp Allergy* 1994; **24**: 318–323.
6. Savolainen J, Kortekangas-Savolainen O, Nermes M, Viander M, Koivikko A, Kalimo K, Terho EO. IgE, IgA, and IgG responses to common yeasts in atopic patients. *Allergy* 1998; **53**: 506–512.
7. Savolainen J, Koivikko A, Kalimo K, Nieminen E, Viander M. IgE, IgA, and IgG antibodies and delayed skin response towards *Candida albicans* antigens in atopic with and without saprophytic growth. *Clin Exp Allergy* 1990; **4**: 332–339.
8. Petitpierre M, Gumowski P, Girard JP. Irritable bowel syndrome and hypersensitivity to food. *Ann Allergy* 1985; **54**: 538–540.
9. Savolainen J, Lammintausta K, Kalimo K, Viander M. *Candida albicans* and atopic dermatitis. *Clin Exp Allergy* 1993; **4**: 332–339.
10. Adachi A, Horikawa T, Ichihashi M, Takashima T, Komura A. Role of *Candida* allergen in atopic dermatitis and efficacy of therapy with various antifungal agents. *Arerugi* 1999; **7**: 719–725 (in Japanese).
11. Back B, Scheynius A, Johansson SG. Ketokonazole in atopic dermatitis: therapeutic response is correlated with decrease in serum IgE. *Arch Dermatol Res* 1995; **287**: 448–451.
12. Tansho S, Abe S, Mizutani S, Ono Y, Takesako K, Yamaguchi H. Protection of mice from lethal endogenous *Candida albicans* infection by immunization with *Candida* membrane antigen. *Microbiol Immunol* 2002; **46**: 307–311.
13. Uchida K, Yamaguchi H, Shabby K. The therapeutic effects of itraconazole, a new triazole antifungal agent, on experimental fungal infections. *Jpn J Antibiot* 1991; **44**: 588–599 (in Japanese).
14. Hirano T, Miyajima H, Kitagawa H, *et al.* Studies on murine IgE with monoclonal antibodies. *Int Arch Allergy Appl Immun* 1988; **85**: 47–54.
15. Whelan WJ, Edwards FE. Isolation of yeast mannan: polysaccharides of Bakers' Yeast. Part IV, Mannan, Stanley Part. *J Chem Soc* 1961; **1**: 29–34.
16. Okubo Y, Ichikawa T, Suzuki S. Relationship between phosphate content and immunochemical properties of subfractions of Bakers' Yeast mannan. *J Bacteriol* 1978; **136**: 63–68.
17. Kobayashi H, Shibata N, Mikobe H, Okubo Y, Suzuki S. Structural study of phosphomannan of Yeast-form cells of *Candida albicans* J-1012 strain with special reference to application of mild acetolysis. *Arch Biochem Biophys* 1989; **272**: 364–375.

Received 10 March 2004

Accepted 22 April 2004