

Nasal vaccination induces the ability to eliminate *Candida* colonization without influencing the pre-existing antigen-specific IgE Abs: a possibility for the control of *Candida*-related atopic dermatitis

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Abstract

In some cases of atopic dermatitis (AD), a possible pathological contribution to disease development by *Candida albicans* (*C. albicans*) has been suggested. AD patients with severe symptoms showing positive capsulated hydrolic carrier polymer radioallergosorbent test (CAP-RAST) against *C. albicans* demonstrated significantly higher levels of serum IgE Abs than did AD patients with mild symptoms. Based on the clinical facts, we have postulated that elimination of *C. albicans* by mucosal vaccination may lead to the restoration of severe symptoms in AD patients. For this purpose, we have developed an allergic murine model. Mice which were systemically challenged with *C. albicans*-associated antigen, manganese superoxide dismutase (MnSOD) or secreted aspartic proteases 2 (SAP2), together with alum, exhibited hyper IgE Abs. Systemically primed mice were then immunized with MnSOD or SAP2 plus cholera toxin (CT) as mucosal adjuvant through the nasal route. Interestingly, nasally immunized mice showed increased levels of *Candida* Ag-specific IgA Ab in fecal and nasal washes as well as in saliva samples but unchanged levels in Ag-specific IgE responses. Consistent with the Ab levels, high numbers of *Candida* Ag-specific IgA Ab-forming cells were induced in mononuclear cells isolated from intestinal lamina propria, nasal passages and salivary glands of nasally vaccinated mice with Ag plus CT. Furthermore, nasal immunization using MnSOD or SAP2 together with CT resulted in the elimination of colonized *C. albicans* from the intestinal tract. These results also suggest a potential role of mucosal vaccination in the control of *C. albicans* in patients with allergic diseases, including AD, although more research is needed to establish this therapeutic approach for mucosal vaccination.

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1. Introduction

Atopic dermatitis (AD) is an inflammatory skin disease associated with atopic diathesis which runs a course of remission and exacerbation [1]. It has been proposed that hypersensitivity to some environmental (e.g. house dust, mites) or food allergens plays an important role in the onset of AD [2]. Since a considerable percentage of AD patients have strong immediate-type skin reactions and specific IgE Ab titers to *Candida albicans* (*C. albicans*), the role of *C. albicans* in the aggravation of AD has been a focus of investigation [1,3,4]. A previous study demonstrated that AD was also often accompanied by the multiplication of *Candida* in the intestinal tract [5]. Consequently, this AD

patient group possessed high levels of *Candida*-specific IgE antibodies (Abs) and was likely to develop serious symptoms because of the simultaneous exaggeration of the Th2-type but the failure of the delayed type reaction to *Candida* [4].

Several studies have reported the relationship between indigenous bacteria and mucosal tissue or skin associated diseases. Our group previously reported that an intestinal microenvironment consisting of foods and bacterial flora such as *Bacteroides vulgatus* is considered to be a causative factor for inflammatory bowel disease [6]. Moreover, the human *Staphylococcus aureus* is a major cause of community- and hospital-acquired skin and respiratory symptoms [7]. It has also been demonstrated that some immunological relationship exists between these two surfaces, skin and mucosal tissues, where low amounts of secretory IgA (S-IgA) Abs were found in tears and on skin surfaces of AD patients [8,9]. Therefore, an obvious and interesting strategy

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would be to induce an effective *Candida*-specific immune response at the mucosal barrier where the colonization of *C. albicans* occurs which may lead to the alteration of disease condition at skin surfaces in AD patients. Anti-fungal drugs have been shown to improve the symptoms of AD patients [10,11]; however, treatment with anti-fungal drugs easily provokes the Jarisch–Herxheimer response [12]. In order to develop an alternative treatment, we have postulated that mucosally induced *C. albicans*-specific immune responses would be able to eliminate *C. albicans* effectively and safely from the gastrointestinal tract of the AD patient.

The purpose of this study was to examine whether the mucosal vaccination can induce *C. albicans*-specific S-IgA responses without affecting the existing hyper-IgE condition. In addition, we have also evaluated the efficacy of the mucosal vaccine at eliminating pre-colonized *C. albicans* from the gastrointestinal tract. Based on the results obtained by this study, we have suggested the possibility that application of nasal vaccination with *C. albicans* antigen such as manganese superoxide dismutase (MnSOD) or secreted aspartic proteases 2 (SAP2) plus cholera toxin (CT) as mucosal adjuvant could be employed for the control of the number of *C. albicans* in patients with severe AD.

2. Materials and methods

2.1. Mice

Balb/c female mice (Japan Clea Co., Tokyo, Japan) at 6–7 weeks of age were housed in the experimental animal facility at the Research Institute for Microbial Disease, Osaka University. All mice received sterilized food (certified diet MF; Oriental Yeast Co., Osaka, Japan) and autoclaved tap water.

2.2. Immunization

In order to establish a sensitized animal model, Balb/c mice were intraperitoneally challenged with MnSOD or SAP2 (100 µg) and an equal volume of alum (PIERCE, IL, USA) at days 0 and 14. Mice were then nasally administered with a 20 µl aliquot (10 µl per nostril) containing MnSOD or SAP2 (10 µg) together with CT (0.5 µg; List Biologic Laboratories, Campbell, CA) at days 63, 70, and 77 for the induction of Ag-specific mucosal immune responses. MnSOD and SAP2 were kindly provided by Takara Shuzo Co. (Kyoto, Japan).

2.3. Detection of Ag-specific Abs

Serum, saliva, fecal extract, and nasal wash were obtained as described previously [13,14]. The Ab titers in serum and external secretions were determined by ELISA as described elsewhere [13,14]. Briefly, plates were coated with MnSOD or SAP2 (100 ng/ml of PBS) and

blocked with 2% bovine serum albumin (BSA) in PBS. After washing, serial dilutions of samples were added in duplicate. Following incubation, the plates were washed and peroxidase-labeled goat anti-mouse μ , γ , or α heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added. The color development was done with 3,3',5,5'-tetramethyl-benzidine (TMB; Moss Inc., Pasadena, CA). Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD at 414 nm of 0.1 greater than background after 15 min of incubation. In no case did non-immunized mice give titers greater than $\log_2$ of 5 in sera and $\log_2$ of 2 in external secretions.

2.4. ELISA for total and Ag-specific IgE Abs

For detection of total and Ag-specific IgE Ab titers in sera, the ELISA method was adopted as described previously [15]. In brief, the immunoplates (Nalge Nunc International, Naperville, IL, USA) were coated with purified rat anti-mouse IgE mAb (R35-72; BD PharMingen, San Diego, CA) and incubated overnight at 4 °C. After blocking with 2% BSA in PBS, serial dilutions of serum samples and mouse IgE mAb (27–74; BD PharMingen) as a standard were added and incubated for 4 h at room temperature. After extensive washing, biotinylated rat anti-mouse IgE mAb (R35-118; BD PharMingen) for total IgE Ab or biotinylated MnSOD or SAP2 for Ag-specific IgE Ab were added, respectively. Then, peroxidase-labeled anti-biotin mAb (Vector Laboratories, Burlingame, CA) was added and the color reaction was developed with TMB (Moss Inc.) for total IgE Ab and with Super Signal ELISA Pico Chemiluminescent Substrate (PIERCE) for Ag-specific IgE Ab.

2.5. Enzyme-linked immunospot (ELISPOT) assay

Spleens were aseptically removed and single cell suspensions were obtained. The nasal passage, salivary gland, and small intestine were carefully excised and mononuclear cells were obtained as described previously [14]. To assess the number of MnSOD or SAP2-specific Ab-forming cells (AFC), an ELISPOT assay was performed [14]. In brief, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with MnSOD or SAP2 (10 µg/ml of PBS) and blocked with 10% FCS in PBS. The blocking solution was discarded, cells at various dilutions were added, and the cell suspensions were incubated for 4 h at 37 °C in 5 % CO₂ incubator. The detection Ab consisted of peroxidase-conjugated goat anti-mouse μ , γ , or α heavy chain-specific Abs (Southern Biotechnology Associates). Following overnight incubation, plates were washed with PBS and spots were then developed by addition of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H₂O₂ (Moss Inc.). Plates were incubated at room temperature for 15–20 min and washed with water, and AFCs were counted with the aid of a stereomicroscope.

2.6. Culture and colonization of *C. albicans* TIMM1768

C. albicans TIMM1768 [16] was kindly provided by the Research Center for Medical Mycology of Teikyo University (Tokyo, Japan) and maintained at 4 °C with monthly transfer on *Candida* isolation agar (Difco, Detroit, MI). For colonization in the gastrointestinal tract, mice were fed with autoclaved tap water containing Ampicillin (1.0 mg/ml, Nakarai tesque, Kyoto, Japan) and Kanamycin (0.2 mg/ml, Nakarai tesque) for disruption of normal microflora from 4 days before inoculation [17]. *C. albicans* TIMM1768 was harvested from the broth cultures by centrifugation, washed in PBS, and diluted to 5×10^6 cells/ml of PBS. Antibiotic-treated mice were challenged intra-gastrointestinally via a plastic catheter (Fuchigami, Kyoto, Japan) in a volume of 200 μ l (1×10^6 cells) per mouse. Colonization of *C. albicans* TIMM1768 was confirmed after 3 weeks of inoculation the method described elsewhere [18].

2.7. Quantification of colony-forming units (CFU) in fecal extracts

Quantification of *Candida* colonies in feces was performed by the plate-dilution method using *Candida* isolation agar supplemented with chloramphenicol (50 mg/ml, Nakarai tesque) [19]. Extracts from fecal samples were obtained by adding weighed pellets to PBS (1 ml per 100 mg of fecal sample) and were then applied to the Agar plate. Following incubation at 37 °C for 48 h, *Candida* colonies were counted. A minimum of five mice per group were tested and each experiment was repeated at least three times.

2.8. Immunosuppression

Mice were treated with cortisone acetate (1.0 mg, Sigma, St. Louis, MO) and cyclophosphamide (6.0 mg, Wako, Osaka, Japan) intraperitoneally as described elsewhere [20].

2.9. Data analysis

Data were expressed as mean $\pm$ S.D. and evaluated by the *t*-test for unpaired samples using a Statview II statistical program (SAS Inc., Cary, North Carolina, USA) designed for the Macintosh computer. *p*-values of less than 0.05 were assumed to be statistically significant.

3. Results

3.1. Nasal immunization by *Candida* Ag plus CT induced Ag-specific IgA Abs without influencing the pre-existing IgE responses in systemically primed mice

In order to investigate the possible role of mucosal vaccination in the control of *Candida*-associated immune

responses, we have first established a murine model in which mice have high levels of *Candida* Ag-specific IgG and IgE Abs in their systemic compartments. For this purpose, groups of mice were challenged with *Candida* Ags, such as MnSOD or SAP2, plus alum, a well-known potent Th2 stimulator [21]. As one might expect, *Candida* Ag-specific IgG and IgE Ab titers in the sera of the challenged mice were significantly higher than those of the naïve PBS group (data not shown). However, we could not find any allergic symptoms (e.g. diarrhea or skin hypersensitivity etc.) in these mice.

We next performed a series of experiments to examine the effect of nasal immunization using MnSOD or SAP2 together with CT as mucosal adjuvant in these pre-sensitized mice with high IgG and IgE Abs. Interestingly, the levels of *Candida* Ag-specific IgA Ab were significantly more increased in sera of mice nasally immunized with MnSOD or SAP2 plus CT than in mice nasally administered with PBS alone (Fig. 1). Consistent with the Ab levels, increased numbers of MnSOD or SAP2-specific IgA AFC were induced in mononuclear cells isolated from spleen of mice nasally immunized with *Candida* Ag plus CT as mucosal adjuvant. However, no significant difference was seen in the IgG Ab titers of serum and IgG AFCs of spleen between the group nasally immunized with Ag plus CT and that immunized with PBS alone. These results clearly indicate that nasal vaccination induces *Candida* Ag-specific IgA Ab responses in the systemic compartment of these pre-sensitized mice.

3.2. Induction of Ag-specific mucosal IgA responses by nasal immunization with *C. albicans* Ag plus CT in systemically primed mice

Since *Candida* invade through and colonize at mucosal tissues [19], it is important to induce effective immune responses at the mucosal surface for the prevention of and/or removal of *Candida* colonization in patients with AD. Therefore, we next examined if nasal vaccination would induce the *C. albicans* Ag-specific Ab responses in the mucosal compartments. Interestingly, significantly higher levels of Ag-specific IgA responses were detected in nasal wash, saliva, and fecal extracts of mice nasally administered with MnSOD or SAP2 plus CT than in those of mice administered with PBS alone (Fig. 2A). Consistent with the Ab levels, high numbers of Ag-specific IgA AFC were detected in mononuclear cells isolated from nasal passages, salivary glands, and the intestinal lamina propria of mice nasally immunized with *Candida* Ag plus CT (Fig. 2B). These results strongly suggest that nasal immunization with *C. albicans*-derived Ags together with CT can induce Ag-specific IgA Ab responses in the systemic and mucosal compartments of pre-sensitized mice with high levels of *Candida* Ag-specific IgG and IgE responses.

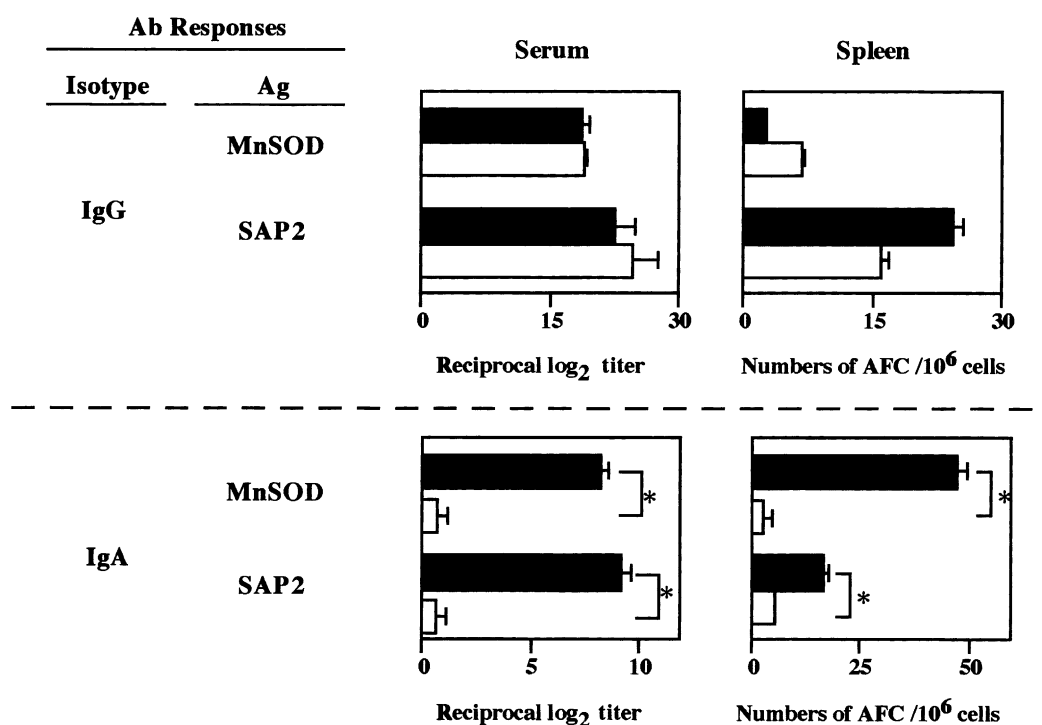


Fig. 1. Nasal immunization with MnSOD or SAP2 together with CT as mucosal adjuvant induces Ag-specific IgA Abs in serum of *Candida* pre-sensitized mice. Mice were intraperitoneally challenged with 100 μ g of MnSOD or SAP2 plus alum at days 0 and 14. Then, mice were nasally administered with 10 μ g of MnSOD or SAP2 together with 0.5 μ g of CT as mucosal adjuvant at days 63, 70, and 77. One week after final immunization, mice were sacrificed. The levels of Ag-specific IgG and IgA Ab in serum and the numbers of Ag-specific IgG and IgA Ab-forming cells (AFC) in spleen of mice nasally immunized with MnSOD or SAP2 plus CT (■) or PBS alone (□) were determined by ELISA and ELISPOT, respectively. Bars represent the mean $\pm$ S.D. in each group. Each group consisted of five mice and the data are representative of three separate experiments. $P < 0.05$ (denoted by *) when compared with PBS group.

3.3. Nasal immunization with *Candida* Ag and CT did not influence the pre-existing levels of IgE Abs

To determine if nasal administration enhances IgE Ab responses, we have assessed both total and Ag-specific IgE Ab levels using ELISA. The levels of both total and Ag-specific IgE Abs in serum were determined before and after nasal immunization (days 62 and 85). As shown in Fig. 3, nasal administration with MnSOD plus CT influenced neither total nor Ag-specific IgE Ab titers. Although two courses of systemic challenge with MnSOD in alum induced high levels of IgE Abs, the nasal immunization did not affect these pre-existing IgE Ab contents. Taken together, our findings indicate that nasal immunization with *C. albicans*-derived Ag together with CT induced mucosal and systemic IgA Ab responses and that it did so without any enhancement of pre-existing IgE Ab levels.

3.4. Ag-specific immune responses induced by nasal immunization with MnSOD or SAP2 plus CT eliminated the pre-colonized *C. albicans* in the gastrointestinal tract and protected mice from infection

In order to assess the efficiency of nasal immunization, a strain of *C. albicans* TIMM1768 that has been shown to

colonize the gastrointestinal tract of mice was used in this study (Fig. 4A). After colonization of the gastrointestinal tract by *C. albicans* TIMM1768, MnSOD- and SAP2-specific IgG and IgA Abs were slightly increased in serum but not saliva or fecal extracts (Fig. 4B and C). Mice were then nasally administered with MnSOD or SAP2 plus CT and *C. albicans* Ag-specific IgG and IgA Ab titers were determined. Interestingly, three rounds of nasal immunization using MnSOD or SAP2 together with CT elicited significant levels of Ag-specific IgA Abs in saliva and fecal extracts (Fig. 4B and C). From 1 week after final nasal immunization, CFU were monitored using the fecal extracts from each group. The numbers of CFU were significantly lower in groups of mice nasally immunized with MnSOD or SAP2 plus CT than in those administered OVA plus CT or PBS alone (Fig. 4D). As one might expect, fecal samples from the non-colonized group did not contain *C. albicans* (data not shown).

It is well established that immunosuppression with cortisone acetate and cyclophosphamide on the *C. albicans*-colonized mice results in penetrative growth by the filamentous form of *C. albicans* in the gastrointestinal tract and in subsequent dissemination to the kidney and liver [20]. To investigate the effects of nasal immunization on the infection of *C. albicans*, mice persistently colonized with

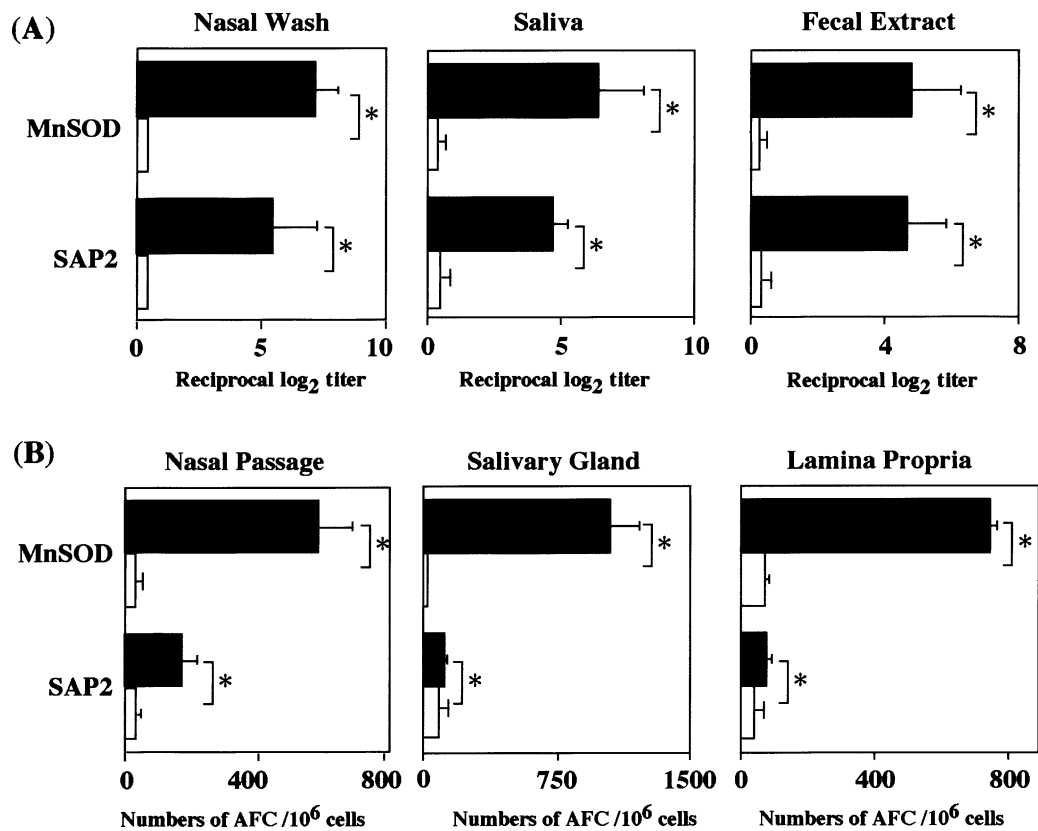


Fig. 2. Nasal immunization with MnSOD or SAP2 together with CT induces Ag-specific IgA Ab responses in mucosal compartments. The levels of Ag-specific IgA Ab in mucosal secretions and the numbers of Ag-specific IgA Ab-forming cells (AFC) in mucosal tissues of mice nasally immunized with MnSOD or SAP2 plus CT (■) or PBS alone (□) were determined by ELISA and ELISPOT, respectively. Detailed immunization methods were described in the legend of Fig. 1. Bars represent the mean $\pm$ S.D. in each group. Each group consisted of five mice and the data are representative of three separate experiments. $P < 0.05$ (denoted by *) when compared with control group.

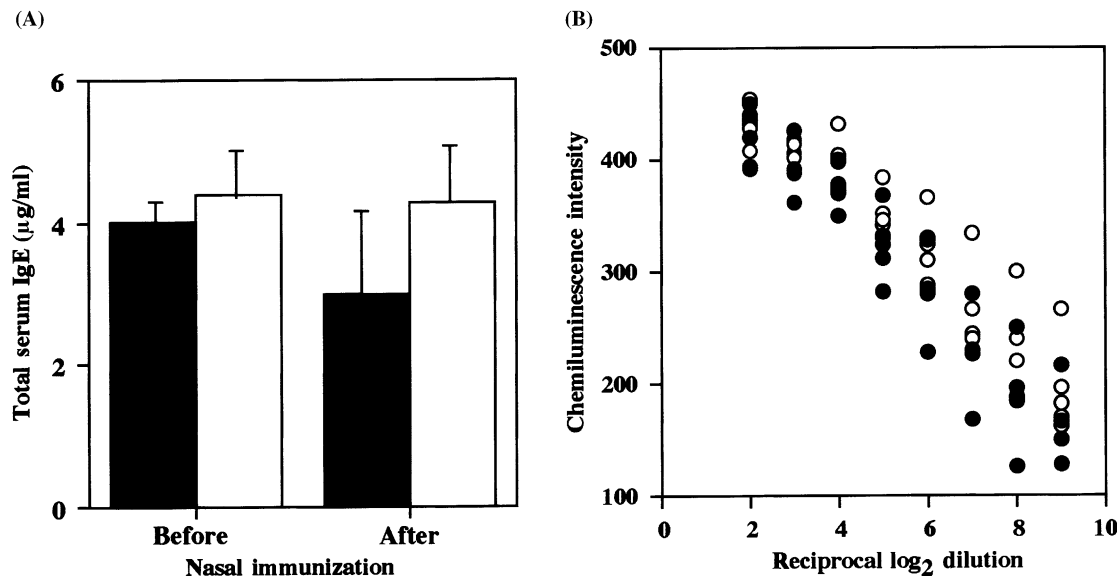


Fig. 3. Nasal immunization with MnSOD and CT did not influence the levels of pre-existing IgE Abs. The IgE Ab levels of both total (A) and MnSOD-specific (B) in serum of mice nasally immunized with MnSOD plus CT (■) or (●) or PBS alone (□) or (○) were determined by ELISA. Detailed immunization methods were described in the legend of Fig. 1. Bars represent the mean $\pm$ S.D. in each group. Each group consisted of five mice and the data are representative of three separate experiments.

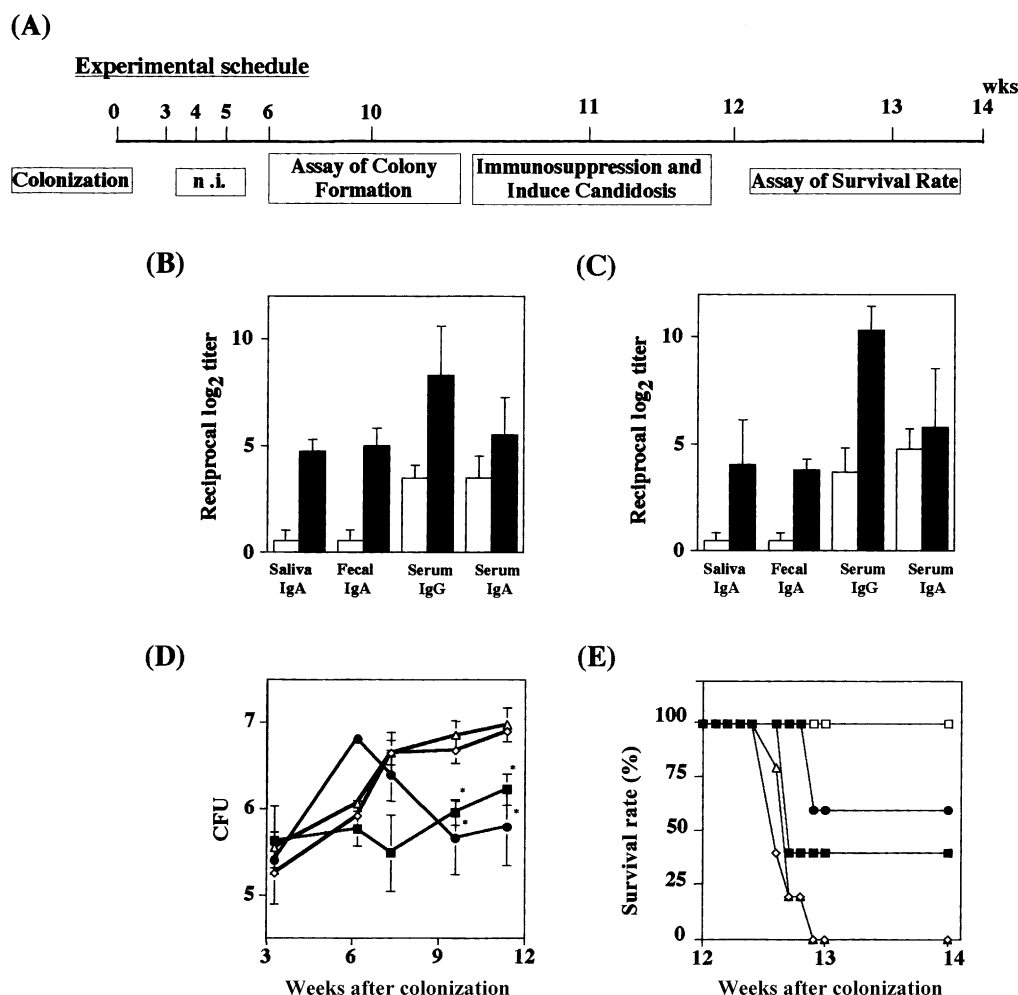


Fig. 4. Nasal immunization with MnSOD or SAP2 plus CT resulted in the removal of *C. albicans* from the intestinal tract of pre-colonized mice. Mice were pretreated for 4 days with drinking water containing Ampicillin and Kanamycin for disruption of normal microflora and then orally inoculated with *C. albicans* TIMM1768 (5×10^6 cells). After 3–5 weeks of inoculation, each group of mice received nasal immunization with MnSOD (B) or SAP2 (C) together with CT. At weeks 3 (□) and 6 (■), serum and mucosal secretions were obtained from each group and then MnSOD- or SAP2-specific IgG and IgA Ab levels were examined by ELISA. In order to assess the influence of nasal immunization on CFU of intestinal *C. albicans* (D), the groups of mice were nasally immunized with MnSOD (■), SAP2 (●) or OVA (◇) together with CT or PBS alone (Δ) and extracts of fecal samples were obtained. Persistently colonized mice were treated with cortisone acetate and cyclophosphamide intraperitoneally at 12 weeks and then survival rates were monitored following nasal immunization [MnSOD (■), SAP2 (●) or OVA (◇) together with CT or PBS alone (Δ)] (E). Groups of uninoculated mice immunosuppressed with cortisone acetate and cyclophosphamide were used as controls (□). All control groups survived. The mice were monitored daily for death. Bars represent the mean $\pm$ S.E. (B,C) and S.E. (D) in each group. Minimum of five mice per group were tested, and each experiment was repeated at least two times. $P < 0.05$ (denoted by *) when compared with PBS alone group.

C. albicans TIMM1768 were immunosuppressed with cortisone acetate and cyclophosphamide (Fig. 4A). Interestingly, groups of mice nasally administered with MnSOD or SAP2 plus CT, which demonstrated low levels of CFU, showed significantly higher survival rate than did mice nasally immunized with OVA plus CT or PBS alone (Fig. 4E). Overall, these results indicate that mucosal immunization with *Candida* Ags, MnSOD or SAP2 together with the mucosal adjuvant CT is capable of inducing effective immunity which leads to the removal of *C. albicans* and protection from candidosis.

4. Discussion

Although it is still controversial whether colonization by *C. albicans* contributes to the symptoms associated with AD [22], it has been reported that an aberrant immune response to *Candida* may be related to the severe stage of AD. A previous study has shown that there is a correlation between the severity of AD symptoms and IgE-RAST titers of *Candida* [23] and that these symptoms can often be mitigated by oral treatments with anti-fungal agents [10,11]. Further, AD patients suffering from a multiplication of

Candida in the intestinal tract [5] have also been shown to exhibit low levels of IgA responses in mucosal secretions (unpublished data from Prof. Ikezawa, Yokohama City University, Medical School, Yokohama, Japan). Therefore, it is possible that an immuno-therapy which could induce mucosal IgA Ab but not IgE Ab responses might prove to be beneficial for the control of intestinal *Candida* in AD patients with low levels of Ag-specific IgA. In this regard, we first established the sensitized murine model with *C. albicans* Ag and examined whether nasal immunization using *Candida* Ag plus CT could induce mucosal immune responses without influencing the pre-existing IgE Abs. Secondly, we adopted the colonization method of *C. albicans* in mouse intestinal tracts and examined whether mucosally induced *Candida* Ag-specific immune responses could remove *C. albicans* from intestinal tracts. The present study has demonstrated that nasal administration of *Candida* antigens together with CT induces IgA production in the mucosal compartments of pre-sensitized mice and protects these mice from colonization and infection by *C. albicans*. These findings provide fresh evidence that nasal vaccination might potentially play an important role in the development of a mucosal immuno-therapy for the control of intestinal microflora in patients with the severe form of AD.

The mucosal immune system employs tightly regulated dynamic mucosal intra- and inter-nets consisting of inductive and effector sites for the induction of an appropriate immunological homeostasis between the host and mucosal environments [24]. The common mucosal immune system (CMIS), which interconnects the inductive (e.g. Peyer's patch or nasopharyngeal-associated lymphoid tissue; NALT) and effector (e.g. intestinal lamina propria and nasal passage) tissues for the induction of the IgA response, is well characterized [25]. Thus, a large number of previous studies have provided evidence pointing to the feasibility of developing a mucosal vaccine by utilizing the CMIS [14,26–28]. Nasal immunization, like other mucosal routes, offers several advantages when compared with parenteral immunization. For example, administration is safer and more comfortable for the patient. Further, lower doses of proteins are required to induce antigen-specific Ab responses when compared with other mucosal routes (e.g. oral), decreasing the cost of vaccination. To elicit mucosal IgA responses comparable to those induced by oral immunization, only 5–10% of the quantity of vaccine is required in nasal administration [25,29]. In addition, the doses used in nasal immunization tend to induce lower total and Ag-specific IgE levels in serum [30]. Taken together, these results suggest that nasal immunization deserves greater attention as a potential new and effective antigen delivery regimen for the control of AD-associated mucosal destruction.

In this study, we demonstrated that significant *Candida* Ag-specific IgA Ab responses were induced in multiple mucosal secretions such as saliva, nasal wash, and fecal extracts in groups of mice nasally given *Candida* Ag (i.e.

MnSOD or SAP2) together with CT as mucosal adjuvant. Moreover, nasal immunization of *Candida* Ag and CT in *Candida* pre-sensitized mice failed to increase total and Ag-specific IgE levels in serum. It has been reported that internal use of anti-fungal drugs leads to the improvement of AD patients suffering with severe symptoms [10]. Total IgE Abs in serum and the *Candida*-specific RAST value were dramatically decreased in these patients, and disease condition was also improved [10]. However, internal use of an anti-fungal has several disadvantages, e.g. destruction of normal microflora, transient aggravation and an efficacy which is only temporary. Mucosal vaccination, however, might be able to rebuild an appropriate mucosal immunity against *Candida* in patients with AD. Thus, it might prevent adhesion of *C. albicans* at the mucosal epithelium. Eventually, immunological homeostasis between intestinal immunity and *C. albicans* could be restored and *C. albicans*-specific IgE Ab could also be decreased for the gradual alleviation of AD symptoms.

Here, we have used two forms of *C. albicans*-associated pathogenic components, MnSOD and SAP2 [18,31–34], as vaccine Ags. In the gastrointestinal tract, the host recognizes *C. albicans* and starts producing reactive oxygen species (ROS) to interrupt fungal growth. Once *C. albicans* invades a host, MnSOD of *C. albicans* plays a pathological role by suppressing ROS activity which had been produced by host neutrophils, macrophages and others [31,32]. Further, SAPs are also widely believed to play a pathological role during the infection process of *C. albicans* [35]. SAP iso-enzymes associated with adhesion of *C. albicans* are expressed in the mucosal tissue [33]. When a mutation was inserted into the SAP region, the infection ability of *C. albicans* was dramatically reduced [36]. These results suggest a critical role for SAP2 in the virulence of *C. albicans* [36]. The fact that MnSOD protects *C. albicans* from the attack of ROS and that SAP2 helps *C. albicans* to adhere to epithelial cells suggests that enhancing levels of anti-MnSOD or anti-SAP2 IgA Abs in the intestinal secretions by nasal vaccination may act to modulate colonization and replication of *C. albicans* in the gastrointestinal tract.

Our present findings demonstrate that nasal vaccination with MnSOD or SAP2 and the mucosal adjuvant CT induces Ag-specific IgA Ab responses which are capable of eliminating *C. albicans* from the intestinal tract. Several recent studies demonstrated that vaccination with *C. albicans* extracts is effective for elimination of *C. albicans* infection [16,37–40]. For example, mice vaccinated with *C. albicans* L-mannan by intravenous injection exhibited fewer vaginal CFU of *C. albicans* [37]. It was also shown that oral candidiasis was effectively suppressed by oral immunization with formalin-fixed *C. albicans* vaccine [39]. In addition, systemic immunization with *C. albicans* membrane Ag in incomplete Freund's adjuvant induced resistance to systemic candidiasis [16]. New results presented by our study offer an alternative immunization regimen for the induction of *Candida*-specific protective immunity.

Vaccination through the mucosal tissues has been shown to be an effective and novel method for the induction of Ag-specific immune responses in both systemic and mucosal compartments [41]. To effectively adopt the mucosal immunization regimen, co-administration of mucosal adjuvant such as CT and heat labeled toxin (LT) have been shown to be necessary [14,26–29,40]. Our present results provide supportive evidence for a new therapeutic approach using nasal administration of MnSOD or SAP2 together with CT for the control of AD. However, CT has potent diarrhea-inducing properties and is unsuitable for use in humans. Thus, the general consensus could be that it is difficult to adapt this immunization regimen for mucosal immuno-therapy for AD patients. In order to overcome this obstacle, our group as well as others have been focusing on developing a mutant form of bacterial toxin which retains adjuvant activity but not toxicity. For example, the newly developed non-toxic mutant CT (mCT) can be considered as a new form of the mucosal adjuvant. Our group has developed mCTs with a mutation in the ADP-ribosyltransferase cleft of CT-A subunit which causes a loss of diarrhoeagenicity but a retention of adjuvant activity when used systemically [29] and nasally [14]. The combination of *pneumococcal* surface protein A (PspA) and mCT induced protective PspA-specific Abs, and therefore should be considered as a candidate adjuvant for vaccinating humans against *pneumococci* [26]. To further explore the potential of AD mucosal immuno-therapy, our next efforts will be focused on determining whether mCTs can be used as a mucosal adjuvant for the induction of *Candida*-specific IgA Ab responses for the elimination of pre-colonized *Candida* from the intestinal tract.

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