**Saccharomyces boulardii** Stimulates Intestinal Immunoglobulin A Immune Response to *Clostridium difficile* Toxin A in Mice

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**Clostridium difficile** is the most common known cause of nosocomial infectious diarrhea in the developed world (18, 19, 27, 28, 37). Pathogenic strains of *C. difficile* produce two large protein exotoxins, toxin A and toxin B (41). Toxin A is a 308-kDa cytoxin and enterotoxin that induces marked intestinal inflammation, fluid secretion, and mucosal injury (12, 23, 41). Toxin B, a 270-kDa protein, stimulates the release of inflammatory cytokines from monocytes and is cytotoxic to mammalian cells (2, 21, 22, 41). Toxin A appears to be the main cause of intestinal injury and inflammation in animal models of *C. difficile* ileocolitis (25, 41). However, toxin B may also cause injury to human colon (43).

Saccharomyces boulardii is a nonpathogenic yeast used to prevent or treat infectious diarrhea of many etiologies (14). In animal studies, *S. boulardii* protects against diarrhea and enterocolitis induced by a variety of enteric pathogens, including *C. difficile* (4, 5, 7–10, 13, 16, 24, 48, 49). In human studies, *S. boulardii* treatment significantly reduced the incidence of simple antibiotic-associated diarrhea (38, 46). *S. boulardii* also reduced the risk of subsequent relapse in patients with a history of multiple episodes of *C. difficile* diarrhea (20, 30, 39, 47).

The host’s immune response to *C. difficile* toxins is now known to play a major role in determining disease expression (24, 26, 29, 31, 32, 33, 50). High titers of serum or intestinal antibodies against toxin A have been associated with asymptomatic carriage of toxigenic *C. difficile* and with shorter and less severe episodes of *C. difficile* diarrhea (32, 33, 34, 40, 45, 51, 52). Buts and colleagues found that *S. boulardii* significantly increased the secretion of immunoglobulin A (IgA) and secretory component in rat small intestine, but they did not study the specificity of the secretory IgA response (3). Based on these findings, we hypothesized that one mechanism whereby *S. boulardii* may protect against infection by *C. difficile* and other enteric pathogens is through a stimulation of the host’s intestinal mucosal immune response (1, 14).

This study was designed to examine this hypothesis by determining whether *S. boulardii* treatment altered serum or intestinal anti-toxin A antibody production in mice exposed to *C. difficile* toxin A. *C. difficile* toxin A was purified from culture supernatants of strain VPI 10463 (American Type Culture Collection, Rockville, Md.) and inactivated by overnight incubation with 1% formaldehyde followed by ultrafiltration (5, 6, 42). For most experiments, BALB/c mice were immunized with formalin-inactivated toxoid A (100 μg) administered by gavage on days 0 and 7, and animals were sacrificed on day 21. *S. boulardii* (Biocodex Laboratories, Montrouge, France) was administered in the drinking water (3 × 10⁸ CFU per ml) from the time of the first oral immunization until the time of sacrifice. In experiments that compared the mucosal adjuvant effects of *S. boulardii* to those of *C. difficile* toxoid A and/or cholera toxin (10 μg; Calbiochem, San Diego, Calif.) administered on days 0, 7, 14, and 21, and the animals were sacrificed on day 35 (15–17, 53). After sacrificing the animals, the small intestine from the pylorus to the cecum was immediately excised and the intestinal contents were harvested by gently wrapping the small intestine around a Pasteur pipette. An equal volume of phosphate-buffered saline containing protease inhibitors was added (Protease Inhibitors-Complete; Boehringer, Mannheim, Germany), the samples were centrifuged, and the supernatants were collected and stored at −80°C.

Antibodies against *C. difficile* toxin A were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (29, 30, 34, 52). Briefly, microtiter plates (Polyisorb; Nunc, Roskilde, Denmark) were coated with purified toxin A (0.5 μg/ml). Intestinal and serum samples were assayed at a 1:50 dilution. Peroxidase-labeled goat anti-mouse IgA (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was used to determine intestinal and serum IgA anti-toxin A. Peroxidase-labeled anti-mouse IgM (Kirkegaard and Perry) and biotinylated goat anti-mouse IgG (Sigma, St. Louis, Mo.) were used to determine serum IgM and IgG anti-toxin A, respectively. Antibody levels are reported as the mean optical density of triplicate samples. To measure total intestinal IgA, microtiter plates (Immunosorp; Nunc) were coated with purified anti-mouse IgA (0.5 μg/ml; Sigma) and intestinal samples were
IgA levels were significantly higher in each of the two *S. boulardii* groups versus the control (*P* = 0.003 by ANOVA). Assayed at a 1:50,000 dilution. Purified mouse IgA (Pharmingen, San Diego, Calif.) was used as the standard.

Statistical analyses were performed using SigmaStat for Windows (version 2.0; Jandel Scientific Software, San Rafael, Calif.). Analysis of variance (ANOVA) on ranks and pairwise intergroup comparisons by Dunn’s method were used. A *P* value of <0.05 was considered statistically significant.

BALB/c mice treated with *S. boulardii* had a small but statistically significant increase (*P* = 0.003) in total IgA levels in their small intestine secretions (Fig. 1). In the *S. boulardii*-fed group, mean IgA levels were 1.9-fold higher than in controls. A similar, 1.8-fold, increase in mean total IgA levels was seen in mice who received both *S. boulardii* and toxoid A. We next examined whether *S. boulardii* treatment could stimulate an intestinal immune response to *C. difficile* toxoid A. Small intestinal IgA anti-toxin A levels were low in control mice, in mice that were exposed to toxoid A (Fig. 2). However, when mice were immunized orally with toxoid A during treatment with *S. boulardii*, there was a marked (4.4-fold) and highly significant (*P* < 0.001) increase in specific small intestinal IgA anti-toxin A levels. In contrast, there was a 4.7-fold increase in the ratio of specific to total IgA (1.09; *P* = 0.01) in mice that were exposed to toxoid A during *S. boulardii* treatment. Thus, *S. boulardii* specifically increases intestinal IgA anti-toxin A levels during mucosal immunization against *C. difficile* toxin A.

These findings suggested that *S. boulardii* may act as a mucosal adjuvant. The mucosal adjuvant activities of cholera toxin...
FIG. 4. *S. boulardii* is more effective than cholera toxin in increasing small intestinal IgA anti-toxin A levels following oral immunization. Mice were immunized with formalin-inactivated toxoid A (TxA) administered by gavage on days 0, 7, 14, and 21 and were sacrificed on day 35. Other mice received cholera toxin (CT; 10 μg) administered by gavage either alone or with toxoid A. Other mice received toxoid A by gavage and *S. boulardii* (Sb) in their drinking water (3 × 10^8 CFU per ml). Small intestinal IgA anti-toxin A levels were measured by ELISA. Results are expressed as optical density (O.D.) units (×1,000) (mean and standard error; n = 9 for each treatment group). IgA anti-toxin A levels were significantly higher in the TxA plus Sb group compared to the control, CT, or TxA plus CT groups (P = 0.003 by ANOVA).

are well recognized (15–17, 53). Therefore, we compared the intestinal IgA anti-toxin A responses following mucosal immunization with toxoid A during treatment with either *S. boulardii* or cholera toxin. As illustrated in Fig. 4, a significant increase in intestinal IgA anti-toxin A was observed only in the *S. boulardii*- and toxoid A-immunized group (3.0-fold increase; P = 0.003). In these experiments we also examined serum antibody levels against *C. difficile* toxin A. As shown in Fig. 5, serum IgA anti-toxin A was highest in the cholera toxin plus toxoid A group, but the differences were not statistically significant (P = 0.35). Serum IgG anti-toxin A was also highest in the cholera toxin plus toxoid A group, but again the differences did not reach statistical significance (P = 0.34). Serum IgM anti-toxin A was highest in the *S. boulardii* plus toxoid A group and in this instance was significantly higher than the control group. Serum IgM anti-toxin A was also somewhat increased in the cholera toxin and toxoid A group, but the increase did not reach statistical significance.

The main finding of this study is that feeding with *S. boulardii* during oral immunization of BALB/c mice with *C. difficile* toxoid A stimulates a specific immune response to toxin A. As reported previously, *S. boulardii* caused a twofold increase in IgA levels in small intestine secretions (3). However, previous studies did not examine the effect of *S. boulardii* treatment on specific immune responses to luminal antigens. We now find that *S. boulardii* induces a specific IgA immune response to toxin A when both are coadministered. The relative increase in specific IgA anti-toxin A production is greater than the observed increase in total IgA levels. This finding is consistent with antigen-specific stimulation of the intestinal mucosal immune system by *S. boulardii*. The mechanisms whereby *S. boulardii* can stimulate intestinal immune responses to coadministered antigens are poorly understood. However, stimulation of a more effective host mucosal immune response to prevalent antigens may be a general mechanism for the efficacy of *S. boulardii* in protecting against a wide variety of enteric disorders (4–11, 13, 35, 36, 42, 44, 48, 49).

There is increasing evidence that the immune response to *C. difficile* toxin A plays an important role in determining the clinical outcome of *C. difficile* infection in humans (24, 31). We recently reported a strong association between high levels of serum IgG anti-toxin A and asymptomatic carriage of *C. difficile* (32). Other clinical studies have shown that a low serum and/or intestinal antibody response to *C. difficile* toxin A is also associated with severe, prolonged, and recurrent *C. difficile* diarrhea (33, 34, 40, 45, 51, 52). Taken together, these studies indicate that an adequate antibody response to toxin A is an important element in asymptomatic carriage of *C. difficile* and in clinical recovery from *C. difficile* diarrhea. Thus, our finding of increased intestinal anti-toxin A levels in *S. boulardii*-treated mice may be directly relevant to the yeast’s protective effects in recurrent *C. difficile* diarrhea and colitis.

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REFERENCES


