**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 cytotoxin 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, 36, 42, 48, 49). In human studies, *S. boulardii* treatment significantly reduced the incidence of antibiotic-associated diarrhea and recurrent *Clostridium difficile* colitis. The administration of *C. difficile* toxoid A by gavage to *S. boulardii*-fed BALB/c mice caused a 1.8-fold increase in total small intestinal immunoglobulin A levels (*P = 0.003*) and a 4.4-fold increase in specific intestinal anti-toxin A levels (*P < 0.001*). Enhancing host intestinal immune responses may be an important mechanism for *S. boulardii*-mediated protection against diarrheal illnesses.

Stimulates Intestinal Immunoglobulin A

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**Saccharomyces boulardii** is a nonpathogenic yeast that protects against antibiotic-associated diarrhea and recurrent *Clostridium difficile* colitis. The administration of *C. difficile* toxoid A by gavage to *S. boulardii*-fed BALB/c mice caused a 1.8-fold increase in total small intestinal immunoglobulin A levels (*P = 0.003*) and a 4.4-fold increase in specific intestinal anti-toxin A levels (*P < 0.001*). Enhancing host intestinal immune responses may be an important mechanism for *S. boulardii*-mediated protection against diarrheal illnesses.

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Toxin A in Mice

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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.) and 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 × 10^8 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.) was 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.

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assayed at a 1:50,000 dilution. Purified mouse IgA (Pharmericagen, 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 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.

We next asked whether the increase in intestinal IgA anti-toxin A levels in *S. boulardii*-treated mice could be the result of a nonspecific stimulation of small intestinal IgA production. We therefore determined the ratio of specific IgA anti-toxin A to total IgA levels in the different groups of mice. As illustrated in Fig. 3, the median ratios of specific to total IgA were similar in the control (0.23) and toxoid A-treated (0.25) mice. The median ratio was somewhat lower (0.16; *P* > 0.05) in mice that were treated with *S. boulardii* but not immunized with toxoid A. This decrease in the ratio results from an increase in total IgA levels in this treatment group that is not associated with a corresponding increase in specific 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
with antigen-specific stimulation of the intestinal mucosal immune system, we observed an increase in total IgA levels. This finding is consistent with previous studies. Specific IgA anti-toxin A production is greater than the observed increase in serum IgA anti-toxin A when both are coadministered. The relative increase in specific immune responses to luminal antigens. We now find that administered antigens are poorly understood. However, stimulation can stimulate intestinal immune responses to coadministration 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). IgA 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 toxin 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. 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. The mechanisms whereby S. boulardii stimulates a specific immune response to toxin A in humans are well recognized.

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. We recently reported a strong association between high levels of serum IgG anti-toxin A and asymptomatic carriage of C. difficile. Other clinical studies have shown that a low serum and/or intestinal antibody response to C. difficile toxin A is associated with severe, prolonged, and recurrent C. difficile diarrhea. 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**


