Prevention of *Clostridium difficile*-induced experimental pseudomembranous colitis by *Saccharomyces boulardii*: a scanning electron microscopic and microbiological study

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(Received 23 November 1989; revised 3 February 1990; accepted 8 February 1990)

The ability of *Saccharomyces boulardii* to protect mice against intestinal pathology caused by toxinogenic *Clostridium difficile* was studied. Different regions of the intestine of experimental mice were prepared for observation by scanning electron microscopy or homogenized for *C. difficile* enumeration and quantification of toxin A by enzyme immunoassay and toxin B by cytotoxicity. The test group was treated for 6 d with an *S. boulardii* suspension in drinking water and challenged with *C. difficile* on day 4. The three control groups were: axenic mice, mice treated with only *S. boulardii* and mice only challenged with *C. difficile*. The results showed that: (i) 70% of the mice infected by *C. difficile* survived when treated with *S. boulardii*; (ii) the *C. difficile*-induced lesions on the small and large intestinal mucosa were absent or markedly less severe in *S. boulardii*-treated mice; and (iii) there was no decrease in the number of *C. difficile* but rather a reduction in the amount of toxins A and B in *S. boulardii*-treated mice.

Introduction

Clostridium difficile is the causative agent of antibiotic-associated diarrhoea and pseudomembranous colitis in human and animals (Bartlett, 1986). This bacterial species produces two antigenically and biologically distinct toxins implicated in the pathogenesis of these diseases (Banno *et al.*, 1984; Bartlett *et al.*, 1978; Lyerly *et al.*, 1986). Toxin A has enterotoxic and weakly cytotoxic activities and causes fluid accumulation and mucosal damage in several animal models (Banno *et al.*, 1984; Mitchell *et al.*, 1986). Toxin B has potent cytotoxic activity and alters the morphology of a variety of cultured cells (Banno *et al.*, 1984; Meador & Tweten, 1988; Sullivan *et al.*, 1982). Toxins A and B act synergistically when injected into the intestine (Lima *et al.*, 1988; Lyerly & Wilkins, 1984). These toxins are lethal for rabbits, mice, hamsters and monkeys (Arnon *et al.*, 1984; Libby

et al., 1982; Lonnroth & Lange, 1983; Lyerly *et al.*, 1985). The pathological effects and death are only observed when both toxins are present (Czuprynski *et al.*, 1983; Lyerly *et al.*, 1985; Wilson *et al.*, 1986).

The lesions produced by these bacteria in man can be reproduced in the well-defined gnotobiotic rodent model. Pseudomembranes have been described in such a model (Czuprynski *et al.*, 1983; Wilson *et al.*, 1986). It has been shown that *Saccharomyces boulardii* has a protective effect against *C. difficile* infections (Corthier *et al.*, 1986; Elmer & McFarland, 1987; Massot *et al.*, 1984; Toothaker & Elmer, 1984), but the direct effect of the yeast on the mucosal surface has never been studied. We have described, however, the kinetics of *S. boulardii* transit in the mouse intestine by scanning electron microscopy (Castex *et al.*, 1987, 1988).

The aim of the work reported here was to study the mucosal lesions produced after inoculation of *C. difficile* into the intestine of gnotobiotic mice and the effect of preventive treatment by *S. boulardii*. The relationship between topographical histology of the mucosa, multiplication of *C. difficile* and the amounts of toxins A and B was investigated.

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Abbreviation: SEM, scanning electron microscopy.

Methods

Clostridium difficile. Strain VPI 10463 (VPI) originally isolated from an abscess, was provided by N. M. Sullivan, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA (Czuprynski *et al.*, 1983).

Experimental schedule and animals. C3H/He axenic mice were reared in Trexler-type isolators fitted with a rapid transfer system (La Calhène, France). They were fed *ad libitum* a commercial diet (RO3-40, UAR, Villemoisson, France) sterilized by gamma irradiation. The experiments were performed over a 6 d period using four groups of 10 mice each; three groups served as controls and one group for the assay. The axenic control mice did not receive any treatment. The *C. difficile* control mice were challenged by the orogastric route on day 4 with 0.5 ml of a fresh culture (approximately 10^8 viable bacteria), and the *S. boulardii* control mice received the yeast in drinking water during the experimental period (Corthier *et al.*, 1986). For the assay group, mice were treated for 6 d with *S. boulardii* and challenged with *C. difficile* on day 4. At the end of the treatment, the intestinal tract was removed. For scanning electron microscopy, the following samples (0.5 cm) were taken: duodenum (0.5 cm after the pylorus), jejunum (11 cm after the pylorus), ileum (1 cm before the caecum), caecum (in the middle) and colon (1 cm after the caecum). The remaining parts were used for bacterial counts and toxin assays. To avoid nonspecific modification of the gut mucosa, intestinal samples were never removed from dead mice but only from healthy or dying mice.

Scanning electron microscopic (SEM) examinations. The specimens were fixed in 1% (v/v) glutaraldehyde for 1 h and washed in 0.2 M-sodium cacodylate buffer, pH 7.2. After stepwise dehydration through a series of graded ethanol solutions, the specimens were coated with gold to a thickness of about 30 nm using a Balzers SCD 020 ion sputter and then examined with a Cambridge Stereoscan model 100 scanning electron microscope (Castex *et al.*, 1987, 1988).

C. difficile counts and toxin assays. The samples were weighed and homogenized. Bacterial counts were performed under anaerobic conditions as described previously (Corthier *et al.*, 1985). Toxin A was measured by an immunoenzymic assay (Mahé *et al.*, 1987). Samples were treated with a trypsin inhibitor (soybean type I-S; Sigma) at a final concentration of 1 mg ml^{-1} . Results were expressed in terms of $\log_{10}[\text{ng (g of gut sample)}^{-1}]$. The cytotoxic effect of toxin B was assayed in tissue culture (Corthier *et al.*, 1985). The cytotoxic titres were transformed into amounts of toxin B expressed in terms of $\log_{10}[\text{ng (g of gut sample)}^{-1}]$ (Corthier *et al.*, 1989).

Results

Survival

All the axenic mice and *S. boulardii* control mice survived. All the *C. difficile* control mice were moribund and would have died from the infection (Corthier *et al.*, 1985) had they not been killed for the SEM and biological examinations of their gut. In the assay group, protected by treatment with *S. boulardii* and challenged with *C. difficile*, seven of the ten mice survived.

SEM examinations

The caecum of the axenic mice showed regular and voluminous folds on a continuous cellular surface (Fig.

1a); in the *S. boulardii* control mice, the caecum had the same appearance but it was covered with yeasts (Fig. 1b). The caecum taken from dying *C. difficile* control mice was completely destroyed and perforated. The cellular surface was disrupted and covered by debris. The bacteria, distributed on the surface singly or in groups, were easily detectable (Fig. 1c). In the mice protected with *S. boulardii* and challenged with *C. difficile*, the mucosal surface of the caecum was almost normal. The cellular surface was not affected and both bacteria and yeasts were visible (Fig. 1d). In the colon, the same features as in the caecum were observed in each group of mice but to a lesser degree. The mucosa of colons taken from dying *C. difficile* control mice had lesions and the bacteria were easily detectable (Fig. 1e). In *S. boulardii*-protected mice, the colon was normal and both bacteria and yeasts were visible (Fig. 1f).

In the small bowel, the same results were obtained with the duodenal, jejunal and ileal mucosal sections; therefore, only the results for the jejunum are discussed. The jejunal mucosa of the axenic mice showed regular tongues and finger-like villi; the villi were covered with yeasts in the *S. boulardii* control mice. In the *C. difficile* control mice, the jejunal mucosa taken from dying mice, even though far from the site of bacterial multiplication, showed lesions, which were mainly localized on the top of the villi (Fig. 1g). *C. difficile* could not be detected. In the *S. boulardii*-protected mice, the jejunal mucosa was normal and neither bacteria nor yeasts were found (Fig. 1h).

Bacterial count and toxin amounts

Numbers of bacteria and amounts of toxins A and B in the digestive tract are presented in Table 1. *C. difficile* counts in the digestive tract were similar in dying control mice and in the assay group (surviving mice). Multiplication of the bacteria occurred mainly in the caecum and the colon. No toxin could be detected in the jejunum in either group of mice. Large quantities of both toxins were present in the caecum and the colon of dying mice, but no (or very little) toxin could be detected in these regions of the gut in protected mice. The reduction of toxin production due to *S. boulardii* treatment ranged from 100- to 1000-fold.

Discussion

The lethal effect of inoculation of gnotobiotic mice with the VPI strain of *C. difficile* has already been reported (Corthier *et al.*, 1985; Czuprynski *et al.*, 1983; Vernet *et al.*, 1989; Wilson *et al.*, 1986); histological staining showed pseudomembranes, extensive inflammation and abrasion of the mucosal surface (Czuprynski *et al.*, 1983;

Fig. 1. Scanning electron micrographs of: (a–d) caeca of axenic control mice (a; bar, 200 μ m), *S. boulardii* control mice (b; bar, 20 μ m), *C. difficile* control mice (c; bar, 50 μ m) and *S. boulardii*-protected mice (d; bar, 50 μ m); (e, f) colons of *C. difficile* control mice (e; bar, 50 μ m) and *S. boulardii*-protected mice (f; bar, 50 μ m); (g, h) jejunum of *C. difficile* control mice (g; bar, 50 μ m) and *S. boulardii*-protected mice (h; bar, 50 μ m).

Table 1. Numbers of bacteria and amounts of toxins A and B in the intestinal mucosa of *S. boulardii*-treated mice challenged with *C. difficile*

Sample*	Bacterial count†	Toxin A‡	Toxin B‡
Jejunum			
Control mice	4.5 ± 0.2	< 1.0	ND
Treated mice	4.6 ± 0.2	< 1.0	ND
Caecum			
Control mice	8.4 ± 0.1	3.0 ± 0.5	3.3 ± 0.1
Treated mice	8.2 ± 0.2	< 1.0	0.9 ± 0.3
Colon			
Control mice	8.3 ± 0.2	2.6 ± 0.5	2.3 ± 0.3
Treated mice	8.0 ± 0.2	< 1.0	ND

* The control mice received only *C. difficile*; they were all moribund at the time of sampling. The *S. boulardii*-treated mice for which results are presented all survived the challenge with *C. difficile*.

† log₁₀ [no. of viable bacteria (g of gut sample)⁻¹] (mean ± standard error).

‡ log₁₀ [ng toxin (g of gut sample)⁻¹] (mean ± standard error). ND, Not detected.

Vernet *et al.*, 1989). The present SEM study permitted a detailed visualization of the villus surface, revealing the complete disruption of the villus structure and release of the internal components in the caecum and colon. It also revealed a previously unknown effect, namely that *C. difficile* caused lesions in the small intestine even though bacterial multiplication was four orders of magnitude lower there than in the caecum or the colon. Although this pathology was slight, it was significant.

Our study confirmed the protective effect of *S. boulardii* treatment on mice challenged with toxinogenic *C. difficile* (Corthier *et al.*, 1986), and showed that this effect was associated with a marked reduction of both toxin A and B production. Similar effects were observed in this animal model using different protective agents such as some bacterial species dominant in the human flora (Corthier *et al.*, 1985) or an adapted diet (Mahé *et al.*, 1987). Until the present study, it was not known whether protected mice develop a transient non-fatal disease. Indeed, one can postulate that, even with protective *S. boulardii* treatment, a slight, undetectable, production of toxins may occur and cause a non-lethal disease. The topographical studies clearly showed the absence of detectable pathological processes in protected mice, suggesting that even if toxin was produced, it remained below the pathological level. The mechanism of *S. boulardii* protection is under investigation.

In conclusion, *S. boulardii* reduced the mortality of mice challenged by toxinogenic *C. difficile*. In protected mice, the intestinal mucosa was practically undamaged and this protection was not due to a decrease in *C. difficile* number but rather to reduced toxinogenesis. It remains to be determined whether *S. boulardii* protection

against *C. difficile* in man is associated with a decrease in the amounts of toxin A and B and intestinal protection as it is in the animal model.

We thank Dr S. L. Salhi for helpful advice in the preparation of this manuscript and Mr G. Nabias for highly qualified assistance in performing the scanning electron microscopic examinations.

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