Role of Candida in Antibiotic-Associated Diarrhea

Robert Krause,1 Egon Schwab,1 Daniela Bachhiesl,1 Florian Daxböck,1 Christoph Wenisch,1 Günter J. Krejs,1 and Emil C. Reisinger2

To quantitatively assess the role of Candida species in antibiotic-associated diarrhea (AAD), stool samples from a total of 395 patients and control subjects were cultured in differential isolation medium: 98 patients had AAD, 93 patients were taking antibiotics but did not have diarrhea (A+D), 97 patients were not taking antibiotics but had diarrhea (A-D), and 107 patients were control subjects (A+D). In addition, secreted aspartyl proteinase (Sap) production was tested. In AAD patients, Candida positivity (77/98) and Candida overgrowth (62/98) were not different from that among A+D patients (75/93 [P = .860] and 52/93 [P = .375], respectively). Candida overgrowth among A-D patients (40/97, P = .003) was less frequent than among AAD patients, but Candida positivity was not different (80/97, P = .612). In control subjects, Candida positivity and overgrowth were less common than in all other groups. Production of Sap did not differ between patients with AAD and control subjects (P = .568 and P = .590, respectively). Data indicate that elevated Candida counts are a result of antibiotic treatment or diarrhea rather than a cause of AAD.

Oral thrush and esophagitis are the most common pathologic manifestations of Candida infections [1, 2]. The role of Candida in antibiotic-associated diarrhea (AAD) has been debated [3–6]. Fecal concentrations of Candida species of ≥105 cfu/mL stool have been considered to cause diarrhea in patients after antibiotic therapy. It has been reported that patients who received antibiotics without developing diarrhea had <102 cfu Candida fungi/mL stool [3].

Secreted aspartyl proteinases (Saps) are important virulence factors produced by Candida species. Saps are involved in the adherence of Candida albicans to human mucosa and in the invasion of human tissue. A correlation between expression of Saps and Candida virulence has been demonstrated in oral candidiasis and Candida vaginitis in human immunodeficiency virus (HIV)-infected patients [7–13]. In Candida-related AAD, active secretion mediated by a fungal toxin or toxinlike substances has been discussed as the pathogenetic mechanism for diarrhea [3, 5, 14, 15]. In C. albicans, 10 different SAP genes have been identified [8]. In Candida tropicalis, weak extracellular proteolytic activity has been observed, whereas Candida glabrata has no proteolytic activity on protein agar [16].

C. albicans is known to produce aspartyl proteinases when grown in medium with protein as the sole source of nitrogen [16–18], and expression of the SAP2 gene is principally responsible for the proteolytic activity observed in most strains growing in the yeast form in vitro [19]. Saps also show proteolytic activity in the gastrointestinal tract; SAP expression in oral candidiasis and degradation of gastrointestinal mucin by Saps has been demonstrated [20, 21]. Degradation of gastrointestinal mucin by enzymes has been implicated as a virulence determinant for a number of enteropathogens (e.g., Vibrio cholerae, Shigella species, Helicobacter pylori, and Yersinia enterocolitica) [22–25]. For C. albicans, which colonizes mucosal surfaces, mucin degradation by SAP could allow for closer approximation to epithelial cells and/or modification of cellular surfaces [20]. Higher levels of secretory Candida acid proteinase were found in children with acute and chronic diarrhea than in healthy control subjects [26].

To elucidate the pathogenetic relevance of Candida species in adult patients with AAD, we performed quantitative stool cultures for Candida species in 395 immunocompetent adults. In addition, the production of Saps was examined.

Methods

Patient selection and collection of stool samples. All patients in a 250-bed medical department were surveyed for diarrhea or for antibiotic treatment in the absence of diarrhea on a daily basis for 11 months. Diarrhea was defined as ≥3 mushy or watery stools per day [27]. Antibiotic treatment was defined as receiving oral or intravenous antibiotics in therapeutic doses for ≥1 day. AAD was defined as ≥3 mushy or watery stools per day during or up to 2
months after antibiotic treatment [27]. Patients were consecutively recruited into 4 study groups until the predetermined numbers necessary for statistical comparison were reached (see statistical analysis below). Patients were included only once during their hospital admission.

Stool samples from all patients with diarrhea were cultured for bacterial pathogens (salmonella, shigella, yersinia, and campylobacter) on selective agar, were examined for ova and parasites, and were tested for Clostridium difficile by culture on selective agar and by use of the rapid C. difficile test (BD). Patients with any of these pathogens were excluded from the study. Thus, 395 patients (195 with diarrhea and 200 without) were included in the study and were assigned to 4 groups: (1) group A Fol D, 98 AAD patients receiving antibiotics and experiencing diarrhea; (2) group A D, 93 patients receiving antibiotics but not experiencing diarrhea; (3) group D, 97 patients not receiving antibiotics but experiencing diarrhea; and (4) group A D, 107 control subjects not receiving antibiotics and not experiencing diarrhea.

The 4 groups did not differ in sex and age distribution. Stool samples from all subjects were collected immediately after a bowel movement and were brought to the laboratory in the same building within 15 min. Samples from stools passed during the night were stored at 4°C and were processed the next morning.

Candida cultures and slide smears. The stool samples were diluted 1:10 with saline, were stirred in a stool homogenizer, and again were diluted 1:1000 and 1:10000 with saline. One hundred microliters of each dilution was transferred onto a CHROMagar (BBL) and was plated evenly with a sterile swab. After incubation at 37°C for 48 h in ambient air, the Candida colonies were counted and classified as C. albicans, C. glabrata, C. krusei, C. tropicalis, or other Candida species, according to the color of the colonies. A colony count $\geq 10^5$ cfu/mL stool was classified as “Candida overgrowth,” according to Danna et al. [3]. A slide smear of every dilution of the first 100 consecutive stool samples was stained with Gram’s stain and was examined by light microscopy for the presence of yeasts and leukocytes.

Detection of Saps. Consecutive Candida isolates from patient groups A D and A D were tested for aspartyl proteinase secretion in bovine serum albumin (BSA) agar (1.17% yeast carbon base [Difco], 0.01% yeast extract [BBL], and 0.2% BSA [Amresco]), as described elsewhere [13]. The medium was adjusted to pH 5.0, was sterilized by filtration (pore size, 0.2 μm), and was added to a stock solution of autoclaved (2%) agar. Proteolysis, indicating enzyme activity, was measured in millimeters and was scored as suggested elsewhere [13]: $–$ or $±$ (no visible or a very limited clarification of the agar around the colony was present), 1+ (appreciable proteolysis $[1–2$ mm] was observed), and 2+ (agar clarification largely exceeded the margin of the colony $[3–5$ mm]). C. albicans strain SC5314 and a SAP2 null mutant (provided by B. Hube, University of Hamburg, Germany) were investigated as controls [19].

Statistical analysis. A number of patients with different Candida counts in different groups were compared by use of the $\chi^2$ test, and yeast counts on slide smears were correlated to the Candida counts from cultures by use of Pearson’s correlation. Differences in Sap production were calculated by use of Student’s $t$ and Fisher’s exact tests. The sample size for the culture test was calculated as 91 evaluable patients, and for the Sap test, it was calculated as 20 evaluable patients per group, with an $α$ of .05 and a power of 0.8 (SigmaStat, version 2.0; Jandel Scientific). Differences with $P < .05$ were considered to be significant.

Results

Candida cultures and slide smears. The results of the Candida cultures are shown in figure 1. In group A D, Candida

Figure 1. Results of Candida counts in stool samples from different study groups: group A D, patients with antibiotic-associated diarrhea who were receiving antibiotics and experiencing diarrhea; group A D, patients receiving antibiotics but not experiencing diarrhea; group A D, patients not receiving antibiotics but experiencing diarrhea; and group A D, control patients not receiving antibiotics and not experiencing diarrhea. Histogram bars (left $Y$-axis) show results of negative cultures (white) and cultures with Candida species, at levels $\geq 10^6$ cfu/mL stool (black) and $<10^2$ cfu/mL stool (gray). Dot plots (right $Y$-axis) next to each of the histogram bars show the cfu/mL frequency distribution.
positivity (in 77/98 patients) and Candida overgrowth (in 62/98 patients) were not significantly different from those in group A'D'. In group A'D', Candida overgrowth was significantly less frequent than in group A'D' (P = .003), but Candida positivity was not significantly different (P = .612). Candida positivity and overgrowth were significantly less common in group A'D' than in all the other groups (Candida positivity: 66/107 patients, P = .013, compared with group A'D'; P = .005, compared with group A'D'; and P = .002, compared with group A'D'); Candida overgrowth: 15/107 patients, P < .001, compared with all other groups; figure 1).

Exploratory analyses using different cutoffs for the definition of Candida overgrowth (10^1, 10^2, 10^3, or 10^4 cfu/mL stool) yielded qualitatively similar results. In all the other groups. C. albicans was the most frequent isolate (216 [55%] of 395 patients) followed by C. glabrata (104 [26%] of 395 patients), C. tropicalis (21 [5%] of 395 patients), C. krusei (11 [3%] of 395 patients), and other Candida species (97 [25%] of 395 patients). Mixed Candida cultures were found in 122 (31%) of 395 patients. Yeast counts on slide smears did not correlate with counts from cultures (r = .27; figure 2).

Proteolytic activity results on BSA agar. Overall, 23 A'D' and 20 A'D' Candida isolates were tested for proteolytic activity in BSA agar. The mean ± SD activity was 1.26 ± 1.06 mm for the A'D' group and 1.42 ± 0.64 mm for the A'D' group. Of the 23 A'D' isolates, 16 had a proteolysis score of 1+ (clarification zone of 1–2 mm) and 1 had a score of 2+ (clarification zone of 3–5 mm), both indicating the presence of proteinase enzyme activity. Of the 20 A'D' isolates, 17 had a proteolysis score of 1+ and 2 had a score of 2+. All C. albicans isolates showed clarification zones on BSA medium (≥1 mm, ≥1+ score). In group A'D', 1 C. tropicalis isolate showed no proteolysis. In group A'D', 5 C. glabrata isolates and 1 C. tropicalis isolate showed no proteolysis. Clarification zones of group A'D' and A'D' were not significantly different when measured either in millimeters (P = .568, Student’s t test) or with the 1+/2+ scoring system (P = .294 and P = .590 for isolates scoring 1+ and 2+, respectively, Fisher’s exact test) [13]. When C. glabrata isolates (which are known to be negative on protein agar) were deleted, there was no significant difference in Sap production between the groups (P = .347).

Discussion

Candida overgrowth has been postulated to cause AAD [3, 6, 14]. We investigated the presence and quantity of Candida species in patients suspected of having AAD, patients without diarrhea but taking antibiotics, patients with diarrhea but not taking antibiotics, and patients without diarrhea and not taking antibiotics (control subjects). A significantly higher proportion of stool samples from patients receiving antimicrobial therapy and experiencing diarrhea (A'D' group) were positive for Candida species and had higher colony counts than did samples from control subjects (A'D' group). In a study by Danna et al. [3], Candida overgrowth (>10^3 cfu/mL stool) was seen in 7 (29%) of 24 AAD patients. In that study, 0 of 24 patients without diarrhea but receiving antibiotics had Candida overgrowth [3]. However, in our study, 52 (56%) of 93 patients with antibiotics but without diarrhea had Candida overgrowth, and no difference was seen, compared with AAD patients. Since we investigated a nearly 4-fold number of patients (93 vs. 24), the differences between our findings and those of Danna et al. [3] may be due to the larger sample size in our study.

To assess the influence of diarrhea on intestinal Candida counts, we also investigated patients not receiving antibiotic therapy but who had diarrhea (group A'D'). In this group, Candida overgrowth (>10^3 cfu/mL stool) was significantly less frequent than in AAD patients, but Candida positivity was not significantly different. Previous studies showed that normal stool flora was markedly decreased in AAD patients. In studies in which the effects of broad-spectrum antibiotics on the composition of intestinal microflora of humans were investigated, a significant inverse correlation between the log of the maximum increase in yeasts and the log of the maximum decrease in anaerobes was observed [3, 14, 28]. Since we found increased Candida positivity in AAD patients, A'D' patients, and A'D' patients, we suggest that the decrease in bacteria in the stools, due to either antibiotic therapy or dilution in diarrhea, allowed Candida species to expand, resulting in increased Candida positivity counts.

In our study, the mean yeast counts on smears stained with Gram’s stain did not correlate with Candida cultures when calculated either with the total yeast numbers on slide smears (r = .27) or with the index suggested by Danna et al. [3] (r = .12). The discrepancy between large numbers of yeastlike cells counted under a light microscope and the poor or absent growth of Candida species has been reported elsewhere [15]. In that study, the authors presumed that a large majority of the
yeasts in stools detected under the microscope were not alive [15]. Our results indicated that great caution should be exercised in interpreting stool samples from patients with AAD and positive for Candida species. The usefulness of analyzing stools for Candida counts by culture or slide smears in these patients is seriously questioned. The absence of leukocytes in samples stained with Gram’s stain was consistent with previous findings [3]. Our results also confirmed that Candida species were part of the normal gut flora.

Active secretion mediated by a fungal toxin or toxinlike substances has been discussed as the mechanism for Candida-related AAD [3, 5, 14, 15]. Since Saps are the major virulence factors in Candida infections [9, 29–33], we investigated these proteinases in vitro. To date, 10 different SAP genes have been identified in C. albicans [8]. Because Sap has been reported to contribute to tissue damage in a model of human oral candidosis [21], these proteinases may also damage intestinal epithelial cells. But in 8 patients suspected of having Candida-related AAD, there was no evidence of colitis or cellular damage [14].

In our study, all investigated C. albicans strains produced Saps, and there was no significant difference between the amount of Saps produced in Candida isolates from AAD patients and control subjects. Higher levels of secretory Candida acid proteinases were found in children with acute and chronic diarrhea than in healthy control subjects [26]. Although Sap levels calculated for the whole group of children with acute or chronic diarrhea were significantly higher than levels in control subjects, this was not the case in single cases. Of 9 Candida isolates, 4 from control subjects (children without diarrhea) had Sap levels comparable to those of children with chronic diarrhea, and 2 of 9 control subjects had levels comparable to those of children with acute diarrhea. Despite these high Sap levels, control subjects did not have diarrhea. This contrasts with findings in previous studies, in which Sap production in symptomatic patients always reached higher levels than in control subjects: Sap production by Candida species from vaginitis patients always exceeded Sap production by Candida from carriers, and Candida strains from oral candidiasis in AIDS patients produced ~8-fold more Sap than did isolates from control subjects [11, 13].

Sap production is coherently indicated by the BSA agar test and ELISA [11, 13]. Moreover, high levels of Sap secreted in vitro and detected by BSA agar or ELISA correlated to increased levels of Sap in vivo (e.g., in vaginal fluids from HIV-positive vaginitis patients) [13]. One can speculate that Candida species temporarily produces high levels of Sap in vivo but loses this capability because of, for example, subculturing. In C. albicans isolated from oral cavities, it was shown that elevated Sap production was a stable characteristic. On the other hand, C. albicans isolates with low proteinase production showed stable, low-level Sap production on repeated subcultures [13].

In summary, the results of our study do not confirm the previously suggested pathogenetic mechanisms of Candida-related AAD [3, 4, 6, 15]. Our data indicate that elevated Candida counts are a result of antibiotic treatment or diarrhea per se rather than a cause of AAD. The production of the major fungal virulence factor Sap in patients with AAD did not differ from that in control subjects, which indicates that this fungal toxin may not be responsible for AAD in adults. Other fungal virulence factors (e.g. phospholipases) warrant further investigation.

References
20. Colma AR, Aumont F, Deslauriers N, Belhumeur P, deRepentigny L. Evi-