CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1995, p. 246–248 Vol. 2, No. 2
1071-412X/95/$04.00+0
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Methods for Detection of an Intestinal Secretory Immunoglobulin A Response to *Candida* spp. and Their Preliminary Application in Human Immunodeficiency Virus-Infected Patients with Chronic Diarrhea

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Received 11 July 1994/Returned for modification 6 September 1994/Accepted 22 November 1994

Six of 11 human immunodeficiency virus (HIV)-infected patients with chronic diarrhea, shedding only *Candida* spp. in their stools, elicited a *Candida*-specific secretory immunoglobulin A response. Similar responses were identified in only 1 of 10 HIV-positive patients with chronic diarrhea but without *Candida* spp. and in none of 10 HIV-negative subjects without diarrhea. *Candida* spp. may play a role in the etiology of chronic diarrhea associated with HIV infection.

Candidal infection of the alimentary tract in the form of stomatitis and esophagitis is a well-recognized occurrence in patients infected with the human immunodeficiency virus (HIV) (14). However, the relevance of candidal infection to the enteropathy and diarrhea often associated with HIV is not completely understood. Despite reports suggesting a role for *Candida* spp. in cases of diarrhea in normal individuals, malnourished children, debilitated hospitalized patients, and elderly patients on antibiotics (3, 4, 7, 10), much of the evidence still remains debatable (2). Recently, *Candida* spp. have been implicated in the etiology of chronic diarrhea and colitis in a patient infected with HIV (5).

The presence or absence of an intestinal secretory immunoglobulin A (sIgA) immune response has been used by our laboratory in the past as an indicator of the possible role of bacteria (6, 16, 17) and viruses (8) as etiologic agents in diarrheal disease. We have provided evidence showing that if an organism is the cause of an enteric infection, it should elicit an sIgA response. The purpose of the present study was to develop the methods required to detect an intestinal sIgA response to *Candida* spp. A preliminary investigation was conducted in which *Candida*-specific sIgA was sought in HIV-infected patients with chronic diarrhea with or without fecal isolation of *Candida* spp.

Subjects studied and extraction of sIgA. Stool specimens were collected from HIV-positive patients with chronic diarrhea attending an outpatient clinic in Harris County, Houston, Tex. (9). Informed consent was obtained from all patients in the study. The study was approved by the Committee for the Protection of Human Subjects of the University of Texas and the Institutional Review Board of the Harris County Hospital District. Eleven individuals who had various *Candida* species as the only organism isolated from their stool samples were identified for this study. As controls, we included 10 HIV-positive patients with chronic diarrhea who did not shed *Candida* spp. in stools. A second control group of 10 HIV-negative individuals without diarrhea was also included for assessment of the specificity of our results. Stool specimens were thawed at room temperature, and sIgA was extracted with 1,1,2-trichlorotrifluoroethane (Sigma Chemical Co., St. Louis, Mo.). Briefly, this procedure consisted of mixing 2 g of stool with 5 ml of phosphate-buffered saline (PBS; 0.01 M, pH 7.2) and incubating the mixture at 56°C for 30 min. Then 5 ml of 1,1,2-trichlorotrifluoroethane was added, and the mixture was vortexed well and centrifuged for 20 min at 4°C. The aqueous phase was stored at −20°C until used for sIgA determinations.

Preparation of *Candida* antigen. *Candida albicans* cells were cultured in Mycobiotic Agar medium (Difco Laboratories, Detroit, Mich.) at 37°C for 48 h. After being washed three times in PBS and centrifuged, the cells were disrupted by pressure disintegration, using a French press cell at 6×10^3 lb/in^2 (11). Cell debris was removed by centrifugation at 20,000 × *g* for 30 min, and the supernatant fraction was stored in aliquots at −80°C.

ELISA. sIgA specific for *Candida* spp. was detected by a direct enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blot (immunoblot). The test wells of flat-bottom polystyrene microtiter plates (Immulon 4; Dynatech Laboratories Inc., Chantilly, Va.) were coated with 100 μl of a 10-μg/ml solution of antigen in bicarbonate buffer (1 M; pH 9.6), and the plates were incubated overnight at 4°C. Each test well had a corresponding blank well coated with bicarbonate buffer alone, in order to ascertain the specificity of the antibodies detected. Plates were blocked with 200 μl of 5% dry milk in PBS for 1 h at 37°C. After the plates were washed once with PBS containing 0.1% Tween 20, 100 μl of twofold serial dilutions of the fecal extracts (starting dilution, 1:2) was added to all wells, and the plates were incubated at 37°C for 2 h. Plates were washed six times; then 100 μl of a 1:2,000 dilution in PBS of a peroxidase-conjugated goat IgG fraction to human sIgA (Cappel Research Products; Organon Teknika Corp., Durham, N.C.) was added to all wells and the plates were incubated for 2 h at 37°C. The washing procedure was repeated, and 100 μl of tetramethylbenzidine substrate (Pierce, Rockford, Ill.) was added to each well; the plates were incubated at room temperature. The reaction was stopped by adding 100 μl of 2 M H_2SO_4 to each well. Color intensity was read at 450-nm wavelength. The optical density of each blank well was subtracted from that of the corresponding well with antigen to give the net optical density. The antibody titer was

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TABLE 1. Intestinal sIgA response against *Candida* spp.

Patient group	No. of patients positive/total tested by:	
	ELISA	Western blot
HIV positive, chronic diarrhea		
Candida spp. in stools	6/11[a,b]	5/11
No organisms in stools	1/10[a]	0/7
HIV negative, no diarrhea	0/10[b]	

[a] $P < 0.05$ by χ^2 analysis.
[b] $P < 0.01$ by χ^2 analysis.

considered to be the highest dilution of the sample giving an optical density twice that of the background. Only fecal extracts exhibiting an antibody titer greater than 1:4 were considered positive for the purposes of this study. All titers given represent the mean of at least two determinations performed on different days.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. The Western blot (immunoblot) procedure employed was that previously described (6). Briefly, *C. albicans* whole-cell extracts (2 mg of protein per ml) were separated on a 4 to 10% discontinuous polyacrylamide gel. Gels were then either stained with Coomassie blue dye or transferred to nitrocellulose. After electrophoretic transfer to nitrocellulose, the unreacted sites were blocked by incubation in 3% dry milk in Tris-HCl buffer at 4°C overnight. The nitrocellulose was then transferred to a Miniblotter 16 (Immunetics, Cambridge, Mass.) and incubated with 120 μl of fecal extract per well for 120 min at 24°C. After gels were washed in Tris-HCl with 0.05% Tween 20, the blots were tested for the presence of antigen-antibody complexes with a biotin-conjugated goat anti-human IgA (alpha-chain specific; Sigma). Horseradish peroxidase-conjugated Extravidin (Sigma) was used to detect the biotinylated secondary antibody. The substrate 0.03% 4-chloro-1-naphthol with 0.01% H_2O_2 was then used to detect the final complex.

Table 1 shows the number of patients in whom an intestinal sIgA response was detected among the three groups tested. Six (54%) of 11 HIV-positive patients with chronic diarrhea shedding only *Candida* spp. in their stools exhibited a positive sIgA response as detected by ELISA. The antibody titers of these positive samples ranged from 1:8 to as high as 1:256. In comparison, an sIgA response against *Candida* spp. was identified in only 1 (10%) of 10 HIV-positive patients with chronic diarrhea who did not shed *Candida* spp. in their stools (sample titer, 1:8) and in none of 10 HIV-negative patients without diarrhea. Western blot analysis confirmed the positive results found by ELISA in five of the fecal extracts, corresponding to HIV-infected patients with chronic diarrhea and fecal isolation of *Candida* spp., and demonstrated that the anti-*Candida* sIgA response was directed to a wide variety of antigens, ranging from approximately 50 to 208 kDa.

Chronic diarrhea and wasting are important manifestations of advanced HIV infection (12). The etiology of diarrhea in HIV-infected patients may relate as much to the immune status of the host as it does to an infecting microorganism. A major problem in HIV-associated infections is the establishment of an etiologic role for an isolated organism in a patient with clinical symptoms. *Candida* sp. is on many occasions the only organism isolated from the intestinal tract of HIV patients with diarrhea (13). While colonization of the gut probably explains most of these cases, at least occasionally, the organism can produce important clinical pathology (5).

It has previously been postulated that the presence of an intestinal sIgA response to an infecting agent can be used as an indicator of its pathogenic role in gastrointestinal disease. To date, most references dealing with *Candida*-specific IgA have looked at the serum, saliva, or cervicovaginal secretions (1, 15, 17), and little is known about the intestinal immune response to *Candida* spp. The direct determination of sIgA from stool extracts is a procedure that has been previously used by our laboratory in studies of pathogenesis and etiology of intestinal infections (6, 8, 16, 17). Our procedure does not require saline purges or intubation for direct sampling of intestinal fluid and because of its simplicity could be used in larger field studies.

In the present study, a modified ELISA technique designed to detect an sIgA immune response to *Candida* spp. found in fecal samples was described. In a preliminary investigation, a majority of the patients (6 of 11) with positive fecal cultures for *Candida* spp. showed an sIgA response, suggesting the presence of an active enteric infection. This sIgA response was directed to a variety of antigens, as evidenced by Western blotting. We speculate that *Candida* spp. may play a role in the etiology of the chronic diarrhea associated with advanced HIV infection. Future study of additional HIV-infected patients with the procedures developed is needed. Additional investigations should be conducted to determine whether HIV-infected patients showing an sIgA response to *Candida* spp. would benefit from anti-*Candida* chemotherapy (3, 4, 10).

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