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Specificity of a Sandwich Enzyme-Linked Immunosorbent Assay for Detecting *Aspergillus* Galactomannan

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The specificity of a sandwich enzyme-linked immunosorbent assay (ELISA) for detecting *Aspergillus* galactomannan was tested with exoantigens of 29 fungi cultured from clinical specimens. Cross-reactivity was observed with *Penicillium chrysogenum*, *Penicillium digitatum*, and *Paecilomyces variotii*. Furthermore, 40 serum samples obtained from bacteremic patients with hematologic malignancies were retrospectively tested by sandwich ELISA. False-positive reactions with the serum were reproducible but did not correspond with the results of culture of specific microorganisms. Moreover, the microorganisms cultured from the blood showed no reactivity by the sandwich ELISA.

Invasive aspergillosis is an opportunistic fungal infection which primarily affects the lungs. Among the patient populations at greatest risk for infection are those with inadequate numbers of circulating neutrophils and those with defective neutrophil function. The crude mortality for patients with invasive aspergillosis is up to 95% (4), which is partly due to the difficulty of diagnosing the infection at an early stage of disease. Definitive evidence of infection can be obtained only by demonstrating invasive growth of hyphae in tissue and culture of *Aspergillus* species from the same tissue specimen. However, the presence of severe thrombocytopenia often precludes the possibility of obtaining a specimen by invasive procedures. Therefore, a multidisciplinary approach is required, in which the results of clinical, radiological, and microbiological examinations are combined. The detection of fungal antigens in serum may contribute to the diagnosis of invasive aspergillosis, but the routine use of antigen detection has been hampered by a lack of sensitivity (3, 11). Recently, a sandwich enzyme-linked immunosorbent assay (ELISA) was developed by Stynen et al. (7). That assay can detect low levels of circulating galactomannan in serum from patients with invasive aspergillosis. The sandwich ELISA uses the rat monoclonal antibody EB-A2 which recognizes the (1→5)-β-D-galactofuranoside side chains of the *Aspergillus* galactomannan (6). The sensitivity of this sandwich ELISA was 90 to 100%, and for some patients circulating galactomannan was detected in the serum even before clinical signs and symptoms suggestive of *Aspergillus* infection became apparent (5, 7-9). A drawback, however, is that up to 8% false-positive reactions were obtained (7-9), which may be due to cross-reactivity with unidentified serum components (8). Although single false-positive reactions can be overcome by testing a series of serum samples (9), persistent positive ELISA results for patients without invasive aspergillosis have been reported (5, 8). False-positive reactions were found to occur, especially within 30 days after bone marrow transplantation (8) and within 10 days after the administration of cytotoxic therapy to patients with hematologic malignancies (8a). During this period patients are often profoundly granulocytopenic and at high risk for both fungal and bacterial infections,

including invasive aspergillosis. Therefore, false-positive ELISA reactions may be due to the presence in the serum of antigens from pathogens other than *Aspergillus* which cross-react with the EB-A2 monoclonal antibody. In the present study, we examined this possibility by analyzing by sandwich ELISA exoantigens from fungi and bacteria cultured from clinical specimens. Furthermore, serum samples from hematologic patients who were bacteremic on the day that the serum sample was obtained were retrospectively tested by sandwich ELISA.

The cross-reactivity of the sandwich ELISA was tested with exoantigens from 29 different fungi which had been cultured from clinical specimens (Table 1). Each fungus, which had been stored at -80°C, was plated onto Sabouraud dextrose (2%) agar and incubated at 30°C for 48 h. One loop of biomass was inoculated into a liquid Sabouraud medium and allowed to grow for 48 h (fast-growing yeasts) or 96 h (other fungi) at 30°C on a rotary shaker at 20 rpm. After 48 or 96 h of incubation, the cultures were inspected for growth and were centrifuged at 2,000 × g for 5 min. The supernatants were filtered through a 0.45-μm-pore-size filter (Millipore S.A., Molsheim, France). Tenfold dilutions ranging from 1:1,000 to 1:1,000,000 were made in distilled water and were tested by the sandwich ELISA. Furthermore, 40 serum samples from febrile neutropenic patients from whom blood for culture had been obtained on the same day and whose blood had become positive on incubation were tested by the sandwich ELISA. These cultures yielded *Staphylococcus epidermidis* (n = 5), *Staphylococcus aureus* (n = 1), *Streptococcus sanguis* (n = 3), *Streptococcus mitis* (n = 5), *Streptococcus oralis* (n = 2), *Streptococcus pneumoniae* (n = 1), *Enterococcus faecalis* (n = 3), *Micrococcus* sp. (n = 1), *Corynebacterium jeikeium* (n = 6), *Pseudomonas aeruginosa* (n = 4), *Pseudomonas cepacia* (n = 1), *Escherichia coli* (n = 4), *Enterobacter cloacae* (n = 2), *Achromobacter xylosoxidans* (n = 1), and the yeast *Candida albicans* (n = 1). The bacteria and yeast cultured from the blood were thought to be primarily responsible for the febrile episode of the patient. Since concurrent infections are common in these patients, the clinical records of each patient were reviewed for the presence of other infections, especially invasive aspergillosis. Furthermore, if a serum sample tested positive by the sandwich ELISA, the microbiological records of the patients were reviewed for test results for additional serum samples which had been collected within 7 days before or after the positive serum sample was obtained and analyzed by sandwich ELISA. The bacterial iso-

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TABLE 1. Reactivity of the sandwich ELISA with exoantigens in a 1:1,000 dilution of the culture supernatant prepared from various fungi

Fungus	Concn by ELISA (ng/ml)	Reactivity compared with that of <i>Aspergillus fumigatus</i> ^a
<i>Aspergillus fumigatus</i>	5.4	100
<i>Aspergillus flavus</i>	5.8	107
<i>Aspergillus niger</i>	6.4	119
<i>Aspergillus terreus</i>	6.6	122
<i>Paecilomyces variotii</i>	5.7	106
<i>Penicillium chrysogenum</i>	6.9	128
<i>Penicillium digitatum</i>	5.8	107
<i>Alternaria</i> species	2.1	39
<i>Rhodotorula rubra</i>	0.7	13
<i>Fusarium solani</i>	0.6	11
<i>Fusarium oxysporum</i>	0.3	6
<i>Trichophyton mentagrophytes</i>	0.4	7
<i>Trichophyton rubrum</i>	0.5	9
<i>Rhizopus oryzae</i>	0.4	7
<i>Absidia corymbifera</i>	0.4	7
<i>Scopulariopsis brevicaulis</i>	0.4	7
<i>Exophiala</i> species	0.4	7
<i>Cladosporium</i> species	0.5	9
<i>Pseudallescheria boydii</i>	0.4	7
<i>Trichoderma viride</i>	0.4	7
<i>Acremonium</i> species	0.4	7
<i>Candida albicans</i>	0.3	6
<i>Candida glabrata</i>	0.3	6
<i>Candida krusei</i>	0.3	6
<i>Candida parapsilosis</i>	0.3	6
<i>Candida tropicalis</i>	0.3	6
<i>Geotrichum candidum</i>	0.4	7
<i>Saccharomyces cerevisiae</i>	0.4	7
<i>Cryptococcus neoformans</i>	0.3	6

^a The reactivity for *Aspergillus fumigatus* is set at 100.

lates were subcultured on sheep blood agar plates. From each isolate dilutions with turbidities equivalent to that of a 0.5 McFarland standard were made and tested by the sandwich ELISA. The sandwich ELISA was performed exactly as described previously (7). Briefly, 300 μ l of each sample was mixed with 100 μ l of treatment solution, and the mixture was subsequently boiled for 3 min. After centrifugation, the supernatant was used for further testing. Fifty microliters of conjugate was added to each well of an antigalactomannan immunoglobulin M-coated microtiter plate (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France); this was followed by addition of 50 μ l of the treated sample. Each plate contained a positive control (5 ng of galactomannan per ml), a weakly positive control (1 ng/ml), and a negative control (0 ng/ml). After 90 min of incubation at 37°C, the plates were washed, 100 μ l of substrate buffer containing *ortho*-phenylenediamine hydrochloride was added to each well, and the mixture was incubated for 30 min at room temperature in the darkness. All assays were performed in duplicate. The galactomannan concentration in the culture supernatants was deduced from the optical density measured at 450 nm and were correlated to a standard curve. The ratio between the optical density of the serum sample and that of the control serum spiked with 1 ng of galactomannan per ml was calculated for each serum sample, and a ratio larger than 1.5 was considered positive, which is recommended by the manufacturer.

The sandwich ELISA may be used to detect circulating *Aspergillus* galactomannan in the serum of neutropenic patients

TABLE 2. Reactivity of the sandwich ELISA with serum samples obtained from febrile neutropenic patients from whom blood samples for culture obtained on the same day were positive for the indicated microorganisms

Microorganism cultured from the blood	No. of serum samples positive/no. of samples tested
Gram-positive organisms	
<i>Staphylococcus epidermidis</i>	2/5
<i>Staphylococcus aureus</i>	0/1
<i>Streptococcus sanguis</i>	0/3
<i>Streptococcus mitis</i>	0/5
<i>Streptococcus oralis</i>	0/2
<i>Streptococcus pneumoniae</i>	0/1
<i>Enterococcus faecalis</i>	2/3
<i>Micrococcus</i> spp.....	0/1
<i>Corynebacterium jeikeium</i>	1/6
Gram-negative organisms	
<i>Pseudomonas aeruginosa</i>	1/4
<i>Pseudomonas cepacia</i>	0/1
<i>Escherichia coli</i>	1/4
<i>Enterobacter cloacae</i>	0/2
<i>Achromobacter xylosoxidans</i>	0/1
<i>Candida albicans</i>	1/1

with fever. However, infections with other fungal or bacterial pathogens may cause a positive ELISA reaction by the production and release into body fluids of exoantigens which cross-react with the EB-A2 rat monoclonal antibody. In preparing the exoantigens of the 29 fungi tested in the present study, we tried to mimic the clinical situation as much as possible by culturing the fungi at 30°C and by analyzing only the culture supernatant without homogenizing each fungus itself. The reactivity of the sandwich ELISA with exoantigens prepared from various fungi is presented in Table 1. As expected, the exoantigens of *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* showed strong reactivities, ranging from 5.4 to 6.6 ng/ml at the 1:1,000 dilution. *Penicillium chrysogenum* and *Penicillium digitatum* showed similar reactivities in the ELISA. The cross-reactivity of EB-A2 observed with exoantigens from *P. chrysogenum* and *P. digitatum* corresponded to that indicated in earlier reports (2, 6). Although these fungi are rarely involved in human disease, they may cause false-positive ELISA reactions as a result of their contamination of clinical specimens (2). We also found that the EB-A2 monoclonal antibody cross-reacted with exoantigens of *Paecilomyces variotii*, which is a fungus highly related to *Aspergillus* organisms (10). In contrast to the findings of Kappe and Schulze-Berge (2), we were unable to find cross-reactivity with antigens from *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Fusarium oxysporum*, *Rhodotorula rubra*, *Exophiala* species, and *Cladosporium* species, which may be due to differences in the preparation of the exoantigens. Finally, an *Alternaria* species was also reactive, but the reactivity was significantly lower than those of the various *Aspergillus* species.

The reactivities of the serum samples from 40 febrile neutropenic and bacteremic or fungemic patients by the sandwich ELISA are presented in Table 2. Eight serum samples (20%) were reactive by the ELISA, but positive ELISA reactions did not correspond with a specific microorganism cultured from the blood. Two patients were suspected of having invasive

TABLE 3. Details of sandwich ELISA reactivity with sequential serum samples from eight patients with a positive ELISA result during bacteremia or fungemia

Patient no.	Blood culture result	<i>Aspergillus</i> infection ^a	ELISA reactivity with the positive serum sample ^b	ELISA reactivity on retesting	ELISA reactivities of sequential serum samples collected between 7 days before and 7 days after the positive sample was obtained ^c
1	<i>S. epidermidis</i>	Unlikely	2.5	2.1	0.7, 0.6, 2.5 , 1.0, 1.4
2	<i>S. epidermidis</i>	Unlikely	2.9	2.3	0.7, 2.9, 2.9 , 1.9, 0.5, 0.5
3	<i>E. faecalis</i>	Possible	2.4	1.6	2.2, 2.5, 2.4 , 1.8, 2.5
4	<i>E. faecalis</i>	Unlikely	2.2	2.2	0.8, 0.8, 2.2
5	<i>C. jeikeium</i>	Unlikely	1.8 (0.9) ^d	2.1 (0.8)	0.2, 0.2, 1.8 , 0.8, 1.2
6	<i>P. aeruginosa</i>	Unlikely	3.7	3.9	1.1, 3.7, 2.0
7	<i>E. coli</i>	Unlikely	1.8	1.5	0.4, 1.8 , 0.4, 1.3
8	<i>C. albicans</i>	Proven	3.3	3.5	0.4, 1.0, 3.3 , 5.8

^a Unlikely infection; no clinical, radiological or microbiological evidence of invasive aspergillosis; possible infection, clinical and radiological evidence of invasive aspergillosis, but negative culture results; proven infection, histopathological evidence of tissue invasion by hyphae and tissue culture yielding *Aspergillus* species.

^b The values represent a ratio calculated by dividing the optical density of the serum sample by that of the control serum spiked with 1 ng of galactomannan per ml. Ratios greater than 1.5 were considered to indicate a positive result.

^c Boldface values represent the reactivities of the serum samples that were obtained during the episode of bacteremia or fungemia.

^d Values in parentheses indicate the reactivities of a second serum sample that was obtained on the same day.

aspergillosis (patients 3 and 8, Table 3), and when the results for these samples were not taken into account, the false-positivity rate was 15%, which is higher than the 8% reported previously (7, 9). The reactivities of the positive serum samples by the sandwich ELISA were found to be reproducible (Table 3) and did not result from the presence of bacteria or *C. albicans* in the blood since the cross-reactivity of EB-A2 with the cultured bacteria and yeast was not observed (data not shown). To examine if the reactivity was present in a single sample or in sequential samples, additional samples were selected from the eight patients. Twenty-seven serum samples which had been collected between 7 days before and 7 days after the positive serum sample was obtained were retrospectively tested by the sandwich ELISA (Table 3). Reactivity was found for a single serum sample from each of four patients (patients 1, 4, 5, and 7) and repeated reactivity was present for serum samples from the remaining four patients (patients 2, 3, 6, and 8) including those suspected of having invasive aspergillosis. A second serum sample was obtained from patient 5 on the day that he was bacteremic, and this sample showed no reactivity by the ELISA (Table 3). These results confirm earlier reports that ELISA reactivity may occur both with single (7, 9) and sequential (5, 8) serum samples from patients without evidence of invasive aspergillosis. Consequently, earlier recommendations to determine the reactivity with additional serum samples (7, 9) may prove useful for some patients, but it will not detect those with false-positive ELISA results with sequential samples. Until the reason for the false-positive reactivity is found, it will be very difficult to distinguish the latter group from those with true antigenemia. However, the course of the ELISA reactivity may help to interpret the ELISA results correctly. Provided that the serum samples are collected regularly, e.g., not less than twice weekly, a sudden rise in the ELISA reactivity would suggest false-positive reactivity (patients 4 and 7), while a more protracted rise would indicate the presence of an *Aspergillus* infection (patient 8). However, the differences in the course of the ELISA reactivity are subtle, and therefore, we recommend that a positive ELISA result should be confirmed by testing additional serum samples by the sandwich ELISA and, if they are found to be positive, obtain evidence of invasive aspergillosis by another diagnostic test or procedure such as early high-resolution computed tomography (12).

The present study suggests that the exoantigens of the tested fungi and bacteria are not responsible for the false-positive reactions by the sandwich ELISA. Other factors may therefore be of importance. This patient population frequently receive transfusions with blood products, but false-positive reactions with antiglobulin serum and blood products has not been found and rules out this factor (8). False-positive antigenemia may be induced by the agents used for immunosuppressive therapy. Indeed, false-positive reactions by the Pastorex *Aspergillus* latex agglutination test, which employs the same monoclonal antibody used by the sandwich ELISA, have been reported with the urine of rats treated with cyclophosphamide (1), and this factor needs to be studied in more detail. Also, severe mucositis, which is present in both bone marrow transplant recipients and patients receiving cytotoxic chemotherapy may play a role by enhancing the resorption of galactomannan or cross-reacting factors from the intestine.

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