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

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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 demonstration of 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 not been hampered by a lack of sensitivity (3, 11). Recently, a sandwich enzyme-linked immunosorbent assay (ELISA) was developed by Strynen 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→3)-B-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. 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.

The specificity of a sandwich 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. 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 inoculated into a liquid Saboraud 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), Streplococcus 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 yeasts 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-
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 μl of each sample was mixed with 100 μl 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 anti-galactomannan immunoglobulin M-coated microtiter plate (Sanofi Diagnostics Pasteur, Marne-la-Coquette, France); this was followed by addition of 50 μl 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 μl 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 supernatant was deduced from the optical density measured at 450 nm and was 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 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 (10). In contrast to the findings of Kappe and Schulze-Berge (2), we were unable to find cross-reactivity with antibodies from *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Fusarium oxysporum*, *Rhodotula 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

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

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.

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.

REFERENCES


