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Comparison of Antigen Detection and PCR Assay Using Bronchoalveolar Lavage Fluid for Diagnosing Invasive Pulmonary Aspergillosis in Patients Receiving Treatment for Hematological Malignancies

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The performance of a sandwich enzyme-linked immunosorbent assay (ELISA) which detects Aspergillus galactomannan (GM) was evaluated in bronchoalveolar lavage (BAL) fluid samples from 19 patients who were treated for hematological malignancies and who were suspected of having invasive pulmonary aspergillosis (IPA). All patients had fever and pulmonary infiltrates on the chest roentgenogram on the day that the BAL fluid was obtained. The ELISA results were compared with the results of culture and Aspergillus genus-specific PCR analysis of BAL fluid samples. ELISA was also performed with serum samples. Aspergillus species were detected by PCR or ELISA with BAL fluid samples from five of seven patients who had radiological evidence of IPA. Serum ELISA results were positive for all patients with ELISA-positive BAL fluid, and for four patients the serum ELISA was positive before the BAL fluid was obtained. PCR and ELISA were positive for 2 and 1 of 10 BAL fluid samples, respectively, obtained from patients without radiological evidence of IPA, and 5 and 2 of 35 BAL fluid samples, respectively, obtained from nonneutropenic patients. This preliminary investigation suggests that GM may be detected by ELISA in BAL fluid samples from patients at risk of IPA, but that monitoring of serum GM levels may allow for the earlier diagnosis of IPA. However, further evaluation in prospective studies is required.

Invasive pulmonary aspergillosis (IPA) is a major threat to leukemic patients with cytotoxic therapy-induced neutropenia and transplant recipients receiving high-dose corticosteroid therapy. Since the diagnosis of IPA in an early stage is seldom possible and the mortality rate is very high (6), successful treatment is directly related to early diagnosis (1). Several methods for obtaining an early and rapid diagnosis of IPA are now under study. Aspergillus DNA can be detected in bronchoalveolar lavage (BAL) fluid by PCR (5, 16, 18, 22, 25), but the numbers of patients and controls tested have been too limited to establish the diagnostic value of this technique. An alternative approach is to detect circulating Aspergillus antigen in body fluids, e.g., serum or urine, by a number of in-house methods (12, 19, 20). The Aspergillus antigen test (Pastorex Aspergillus; Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) is commercially available and uses the rat monoclonal antibody EB-A2 to detect galactomannan (GM), a polysaccharide antigen of Aspergillus species (13). This antigen test has been evaluated in several institutes (4, 8, 9, 26, 29), but it has been found to lack sufficient sensitivity (8, 9, 26, 29). A recently developed sandwich enzyme-linked immunosorbent assay (ELISA) allowed the detection of low levels of circulating Aspergillus GM in sera from patients at high risk of IPA, but the increase in sensitivity was also associated with false-positive results in up to 8% of the serum samples (21, 27). Since IPA is predominantly a pulmonary infection in immunocompromised patients, and Aspergillus fumigatus is known to release GM into the culture medium when it is grown in vitro (13), detectable antigen levels may be present in BAL fluid samples from patients suspected of having IPA and therefore may be of use for the diagnosis of IPA.

We therefore investigated the use of the sandwich ELISA to detect GM with BAL fluid samples which had been collected from patients at high risk and from patients at low risk of IPA. The results were compared with those obtained by culture and Aspergillus genus-specific PCR amplification and were related to the clinical outcome.

MATERIALS AND METHODS

Patients. During a 12-month period 19 consecutive BAL fluid samples had been collected from patients who were neutropenic as a result of cytotoxic therapy or from whom circulating granulocytes were recovered. In each patient a bronchoalveolar lavage was performed because fever (temperature, >38.3°C) persisted despite treatment with broad-spectrum antibacterial agents and pulmonary infiltrates developed on the chest roentgenogram. The BAL fluid sample was obtained by wedging a flexible bronchoscope in the area of maximal roentgenographic involvement, after which 150 ml of sterile 0.9% saline was instilled and approximately 100 ml was recovered by suctioning. The medical, histopathological, microbiological, and radiological records of all patients were reviewed for evidence of IPA or other respiratory disease. Since a definitive diagnosis of IPA is very difficult to establish in this patient group, the patients were allocated to three categories by review of the appearance of the pulmonary infiltrates on the chest roentgenogram by two physicians who were blinded to the medical histories of the patients. The patients were allocated to one of the following groups: A, probable IPA (n = 7), with the chest roentgenogram showing focal nonanatomical infiltrates or any evoluting lesion; C, unlikely IPA (n = 10), with the chest roentgenogram showing diffuse infiltrates characterized by nodular, reticular, or reticulonodular lesions which were distributed throughout both lung fields or anatomic focal infiltrates (13); and B, possible IPA (n = 2), which included...
patients with infiltrates on the chest roentgenogram who could not be classified into group A or C. Of course, the reliability of these definitions is limited because the roentgenographic pattern is not always specific for the infectious etiology of the infiltrates. Furthermore, 35 BAL fluid samples were collected from nonneutropenic patients who were at low risk for IPA.

**Sample collection.** BAL fluid samples were routinely examined for the presence of bacteria, viruses, fungi, and parasites. A total of 3 to 10 ml of each sample was stored at -80°C until analysis by ELISA and PCR. The microbiological records were reviewed for serum samples which had been collected from each patient between 6 weeks before and 2 weeks after the BAL fluid sample was obtained. These samples had also been stored at -80°C until testing by ELISA was performed.

Aspergillus culture. Routine culture for fungi was performed after centrifugation of the BAL fluid sample at 1,500 x g for 10 min. The pellet was plated onto Sabouraud glucose (2%) agar and was cultured for 3 days at 28 and 4°C. Aspergillus species were identified by one technician (A.J.M.V.L.R.) by their cultural characteristics and the morphologies of their conidiophores and conidia.

**PCR amplification.** After thawing, the BAL fluid samples were centrifuged at 1,500 x g for 10 min. The supernatant was separated from the pellet, leaving a residue of 0.5 ml in which the pellet was resuspended. PCR analysis was performed as described previously (16, 25). Briefly, high-frequency shaking was performed to achieve complete cell destruction; this was followed by purification of nucleic acids by guanidinium thiocyanate-containing lys buffer and silica particles. A hot-start PCR was performed with two Aspergillus genus-specific primers. This PCR assay can detect a wide range of the Aspergillus species involved in human disease specifically when it is combined with Southern blot hybridization and restriction enzyme analysis (16).

**ELISA** antigen detection and PCR of BAL fluid. One research group has previously described the sandwich ELISA used to detect Aspergillus GM in serum samples and BAL fluid supernatant was performed exactly as described previously (21). Saline (0.9%) and culture- and PCR-negative BAL fluid supernatant from patients at low risk of IPA containing a range of dilutions of GM (range, 0.25 to 10 ng/ml) and a negative control BAL fluid sample were included in each test. All serum and BAL fluid samples were tested in duplicate by one technician who was unaware of the clinical data. The microtiter plates were prepared by the manufacturer (Serion Pneum Diagnostics), and the coating conditions of the plates were proprietary. However, since the coating conditions of the EB-2 monoclonal antibody on the microtiter plate were different from those reported previously (21), lower optical density values were found.

### RESULTS

**Aspergillus ELISA.** Different concentrations of GM (range, 0.25 to 10 ng/ml) were added to 0.9% saline and culture- and PCR-negative BAL fluid samples from 35 control patients are given in Table 1. Serum samples were available from the period before the bronchoscopy was performed for four patients, and GM could be detected up to 30 days before the BAL fluid sample was obtained (patient A5, Table 2). Shortly after bone marrow transplantation during severe neutropenia, patient A5 developed fever and sinusitis. The administration of ciprofloxacin and amoxicillin-clavulanic acid did not result in clinical improvement, and amphotericin B (1 mg/kg of body weight per day) was added to the regimen. During recovery of the granulocytes, a chest roentgenogram showed the development of a new infiltrate. Although ultrasound of the abdomen suggested acute cholecystitis, a definitive diagnosis was not made by PCR and ELISA of BAL fluid samples were positive, and GM was retrospectively detected in the serum at the time that the sinusitis first became apparent.

Both PCR and ELISA were positive for one of two BAL fluid samples from patients with possible IPA, but serum samples were not available for analysis by the ELISA. The PCR was positive for BAL fluid samples from patients C1 and C2, who had no evidence of the presence of IPA, and GM was not detected in the BAL fluid or serum samples from these patients.

### DISCUSSION

The diagnosis of IPA in an early stage of the disease is of great importance since early antifungal treatment is associated with an improved outcome (1). The early diagnosis of IPA is difficult, and a definitive diagnosis is often made at autopsy. The presence of severe thrombocytopenia in neutropenic patients limits the use of invasive diagnostic procedures to obtain a specimen for culture and histopathological examination. BAL has been advocated as a safe procedure for obtaining a specimen for the diagnosis of IPA in immunocompromised patients with pulmonary infiltrates. Although the recovery of Aspergillus species from BAL fluid is highly indicative of IPA, the diagnostic yield from culture of BAL fluid samples is only 30% (24). Therefore, attention has focused on other techniques for the early diagnosis of IPA, such as Aspergillus-specific PCR of BAL fluid (5, 16, 18, 22) and the detection of circulating Aspergillus antigen in serum or urine (4, 8, 9, 12, 19–21, 26, 29). Little is known about the utility of Aspergillus antigen detection in BAL fluid. One research group has pre-
result obtained from the use of a BAL fluid sample can be due to the presence of contaminating conidia without any mycelial development (see, for example, data for patients C1 and C2). Indeed, previous studies found a rate of 15% positive PCR amplification in BAL fluid samples from nonneutropenic patients (22, 25). The occurrence of a negative PCR result for patient A6, whose BAL fluid and serum samples were GM positive and who had radiological and clinical evidence of IPA, could be due to the presence of inhibitors of the PCR in the BAL fluid (23).

False-positive results were also obtained by the sandwich ELISA. The reasons for the false-positive reactions are unknown, but it has been shown that molecules which are responsible for the false positivity appear to mimic the epitope recognized by anti-GM monoclonal antibodies (21). In 30 culture- and PCR-negative BAL fluid samples from nonneutropenic patients, a 7% false-positive rate was found, which is similar to the 8% false-positive rate found in serum (21). A 10-fold concentration of the samples by acetone precipitation resulted in a false-positive rate of 27% (data not shown). Analysis by PCR and ELISA can be done in less than 24 h (PCR, 24 h; ELISA, 4 h), and the results can be forwarded to the clinician on the day following the BAL.

An excellent correlation was found between serum and BAL fluid ELISA results, and GM was detected in the serum even though the BAL fluid was negative in only a few cases. For this reason, it was interesting to compare DNA and GM detection, since these two assays have a different target. In the case of diagnosis by PCR, a positive result obtained from the use of a BAL fluid sample can be due to the presence of contaminating conidia without any mycelial development (see, for example, data for patients C1 and C2). Indeed, previous studies found a rate of 15% positive PCR amplification in BAL fluid samples from nonneutropenic patients (22, 25). The occurrence of a negative PCR result for patient A6, whose BAL fluid and serum samples were GM positive and who had radiological and clinical evidence of IPA, could be due to the presence of inhibitors of the PCR in the BAL fluid (23).

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An excellent correlation was found between serum and BAL fluid ELISA results, and GM was detected in the serum even
before the BAL was performed. Since a bronchoscopy was performed only after pulmonary infiltrates were present on the chest roentgenogram, GM may be detectable in the serum before infiltrates become visible on the plain chest film. It has been shown that in neutropenic patients abnormalities are difficult to detect on plain chest film (17) and that other radiologic methods, such as high-resolution computed tomography, are more sensitive for the detection of pulmonary infiltrates in these patients (10, 11, 28) and may even be used for therapeutic monitoring (28).

This preliminary investigation suggests that both Aspergillus genus-specific PCR and sandwich ELISA might be beneficial for use in the early diagnosis of IPA in patients with hematologic malignancies. The ELISA is relatively easy to perform, and the results are available within 4 h. Since false-positive results may be found by ELISA of the serum, detection of Aspergillus species in BAL fluid by ELISA or PCR may provide additional evidence for the presence of IPA. The results of the present study also suggest that in some patients GM antigenemia may be detected in serum before pulmonary infiltrates are visible on the chest roentgenogram. However, carefully designed prospective studies need to be performed in order to evaluate the diagnostic value of these assays. We are prospectively monitoring serum GM levels by ELISA in patients during cytotoxic therapy-induced neutropenia. When GM is detected for the first time, confirmation is sought by ELISA with additional serum samples, high-resolution computed tomography scan, and where possible, bronchoscopy and PCR analysis of BAL fluid. Once the presence of GM is confirmed or the signs or symptoms pathognomonic for IPA have developed, treatment with antifungal agents is started preemptively. By following these guidelines, the diagnosis and treatment of IPA may be possible at an early stage of infection.

REFERENCES


