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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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 antigens 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 bronchoscopy 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 radiographic 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 evolving lesion; C, unlikely IPA (n = 10), with the chest roentgenogram showing diffuse infiltrates characterized by nodular, reticular, or reticular nodular lesions which were distributed throughout both lung fields and anatomical focal infiltrates (15); and B, possible IPA (n = 2), which included

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Aspergillus obtint. These samplrs had also been stored at −80°C until testing by ELISA for the presence of bacteria, viruses, fungi, and parasites. A total of 5 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.R.M.) by their cultural characteristics and the morphologies of their conidia and conidiophores.

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 guanidium thiocyanate-containing lysis 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. The sandwich ELISA used to detect Aspergililus GM in serum samples and BAL fluid supernatants was performed exactly as described previously (21). Saline (0.9%) and culture- and PCR-negative BAL fluid supernatants 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 (Sanofi Pasteur Diagnostics), and the coating conditions of the plates were proprietary. However, since the coating conditions of the ELISA were the same, the microtiter plate was 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 low-risk patients and were analyzed by ELISA. The degree of increase in the optical density values of 0.9% saline spiked with increasing concentrations of GM was directly proportional to that found with culture- and PCR-negative BAL fluid samples spiked with the same concentrations of GM (data not shown). The mean optical density values of the 30 BAL fluid samples from nonneutropenic patients with negative fungal culture and PCR results were used to study the ELISA background. There was a Gaussian distribution of the optical density values, allowing a cutoff value of 0.17 to be calculated from the mean optical density value (mean optical density value, 0.09; range, 0.056 to 0.146) plus 4 standard deviations. The optical density of control serum spiked with 1 ng of GM per ml was used as cutoff value for serum (optical density value, 0.17) (21). The results of Aspergililus genus-specific PCR and ELISA for the BAL fluid samples from 19 patients with hematological malignancies and the BAL fluid samples from 35 control patients are given in Table 1.

Clinical data. The characteristics of the 19 patients with hematological malignancies and the results of culture, PCR, and ELISA are given in Table 2. Five of seven BAL fluid samples from patients with probable IPA were positive by PCR and ELISA. GM was detected in serum samples from all patients with an ELISA-positive BAL fluid sample. Moreover, 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 nodule in the right lung. Although histological examination and BAL fluid remained negative, 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. GM was detected in a PCR-negative BAL fluid sample from patient C3, and two of seven serum samples were also found to be positive. The two ELISA-positive serum samples were obtained on the day that the BAL was performed and 1 day thereafter, respectively. Five consecutive serum samples obtained during 2 weeks following the BAL were all negative, even though severe neutropenia persisted and at the time of the sereconversion only a loading dose of 10 mg of amphotericin B had been administered. Treatment with ciprofloxacin and amphotericin B (1 mg/kg/day) for 2 weeks resulted in resolution of the pulmonary infiltrates.

Nonneutropenic patients. PCR analysis of 35 BAL fluid samples from nonneutropenic patients revealed positive amplification reactions for five samples which were all negative by ELISA (25). The ELISA was positive for 2 of 30 PCR-negative BAL fluid samples (Table 1).

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-

TABLE 1. Results of Aspergillus genus-specific PCR amplification and sandwich ELISA of BAL fluid samples obtained from 19 patients with hematological malignancies and 35 nonneutropenic patients

<table>
<thead>
<tr>
<th>Microbiological analysis result</th>
<th>Hematologic patients</th>
<th>Nonneutropenic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR positive, ELISA positive</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>PCR positive, ELISA negative</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>PCR negative, ELISA positive</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PCR negative, ELISA negative</td>
<td>9</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>35</td>
</tr>
</tbody>
</table>
viously described the use of a radioimmunoassay to detect an uncharacterized purified A. fumigatus carbohydrate in BAL fluid (21, 30, 31). This radioimmunoassay detected Aspergillus antigen in 91% of the BAL fluid samples from rabbits with IPA, and in 27% of these animals antigen was detected in BAL fluid only and not in serum (2). The radioimmunoassay was also used to detect Aspergillus antigen in BAL fluid samples from nine immunocompromised patients with pulmonary infiltrates, and two patients were found to be positive (3). GM was detected by a latex agglutination test (Pastorex Aspergillus) in BAL fluid samples from three patients with AIDS and IPA (14). However, GM could not be detected by the same test under laboratory conditions in BAL fluid samples from 14 untreated rabbits with IPA, while serum samples from 10 (71%) animals were positive (7). Although the present study was limited by the difficulty in reliably classifying patients with IPA, GM was detected by sandwich ELISA in BAL fluid samples from five of seven patients with radiological evidence of IPA, and therefore, this test may be promising for use in the diagnosis of this infection.

The increase in the level of sensitivity required to diagnose early IPA, however, is hampered by an increase in the number of false-positive results. 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).

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

**TABLE 2. Characteristics of 19 patients with hematological malignancies and results of analysis of BAL fluid samples by culture and Aspergillus genus-specific PCR and BAL fluid and serum samples by ELISA**

<table>
<thead>
<tr>
<th>Category</th>
<th>Patient no.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Underlying disease and/or condition(s)</th>
<th>BAL fluid analysis</th>
<th>No. of samples positive by serum ELISA</th>
<th>Time between first positive serum and BAL</th>
<th>Granulocyte count (10^5/μl)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>M</td>
<td>43</td>
<td>NHL</td>
<td>Positive A. fumigatus</td>
<td>+ 0.581 (+)</td>
<td>3/5</td>
<td>-4</td>
<td>3.6 Deceased</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>36</td>
<td>NHL</td>
<td>Positive A. fumigatus</td>
<td>+ 0.278 (+)</td>
<td>3/4</td>
<td>-3</td>
<td>5.8 Deceased</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M</td>
<td>48</td>
<td>HL</td>
<td>Positive A. fumigatus</td>
<td>+ 0.129 (+)</td>
<td>0/3</td>
<td>-2</td>
<td>7.5 Survived</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>M</td>
<td>54</td>
<td>AML</td>
<td>Positive A. fumigatus</td>
<td>+ 1.325 (+)</td>
<td>1/1</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>M</td>
<td>22</td>
<td>AA, BMT</td>
<td>Positive A. fumigatus</td>
<td>+ 0.370 (+)</td>
<td>25/28</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>M</td>
<td>58</td>
<td>MDS, BMT</td>
<td>Negative</td>
<td>- 0.247 (+)</td>
<td>3/11</td>
<td>-11</td>
<td>2.1 Survived</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>M</td>
<td>20</td>
<td>Myelofibrosis</td>
<td>Positive A. fumigatus</td>
<td>- 0.095 (-)</td>
<td>NA</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>M</td>
<td>59</td>
<td>AML</td>
<td>Negative</td>
<td>+ 0.736 (+)</td>
<td>NA</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>68</td>
<td>NHL</td>
<td>Negative</td>
<td>- 0.113 (-)</td>
<td>NA</td>
<td>9.2 Deceased</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>M</td>
<td>48</td>
<td>AML, BMT</td>
<td>Negative</td>
<td>+ 0.152 (-)</td>
<td>0/1</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>36</td>
<td>AML</td>
<td>Negative</td>
<td>+ 0.122 (-)</td>
<td>NA</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M</td>
<td>34</td>
<td>CML, BMT</td>
<td>Negative</td>
<td>+ 0.220 (-)</td>
<td>0/2</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>F</td>
<td>52</td>
<td>MM</td>
<td>Negative</td>
<td>- 0.093 (-)</td>
<td>0/1</td>
<td>3.8 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>F</td>
<td>43</td>
<td>ALL, BMT</td>
<td>Negative</td>
<td>- 0.075 (-)</td>
<td>0/5</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>F</td>
<td>40</td>
<td>AML</td>
<td>Negative</td>
<td>- 0.077 (-)</td>
<td>0/1</td>
<td>2.1 Survived</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>F</td>
<td>44</td>
<td>MM</td>
<td>Negative</td>
<td>- 0.087 (-)</td>
<td>0/1</td>
<td>3.5 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>F</td>
<td>62</td>
<td>CLL</td>
<td>Negative</td>
<td>- 0.079 (-)</td>
<td>NA</td>
<td>2.0 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>F</td>
<td>56</td>
<td>AML</td>
<td>Negative</td>
<td>- 0.114 (-)</td>
<td>0/2</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>F</td>
<td>72</td>
<td>AML</td>
<td>Negative</td>
<td>- 0.096 (-)</td>
<td>0/2</td>
<td>&lt;0.1 Deceased</td>
<td></td>
</tr>
</tbody>
</table>

* a Categories: A, probable IPA, with the chest roentgenogram showing focal nonanatomical infiltrates or any cavitating lesion; B, possible IPA in patients with infiltrates on the chest roentgenogram who could not be classified into group A or C; C, IPA unlikely, with the chest roentgenogram showing diffuse infiltrates.

* b M, male; F, female.

* c NHL, non-Hodgkin's lymphoma; HL, Hodgkin's lymphoma; AML, acute myeloid leukemia; AA, aplastic anemia; BMT, bone marrow transplantation; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; MM, multiple myeloma; CLL, chronic lymphocytic leukemia.

* d Patients from whom at least one serum sample was taken within 6 weeks before the BAL fluid sample was obtained. Values are numbers of days between the time the first serum sample was found to be positive by ELISA and the date of bronchoscopy.

* e Optical density value; plus and minus signs indicate interpretation of results.

* f NA, not available.

* g Microscopic detection with Giemsa staining.
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 antigenuria compared to antigenemia in bone marrow transplant recipients.

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