Frequent False-Positive Results of Aspergillus Latex Agglutination Test

Transient Aspergillus Antigenemia during Neutropenia

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BACKGROUND. Two serologic assays, Aspergillus latex agglutination testing (LA) and plasma (1→3)-β-D-glucan (BDG) measurement, are used when invasive pulmonary aspergillosis (IPA) is suspected. Despite the high specificity of these assays, false-positive results are frequent for neutropenic patients. This study was conducted to evaluate the efficacy of LA and BDG and to investigate the cause of the false-positive results.

METHODS. Eighty-eight consecutive patients with hematologic malignancies who underwent intensive chemotherapy were tested weekly with LA and BDG.

RESULTS. Sixteen of 88 patients were diagnosed as having IPA. The sensitivity, specificity, and positive predictive values were 23%, 98%, and 64% for LA and 27%, 88%, and 52% for BDG, respectively. Of 11 patients who became positive for LA only during neutropenic periods, 2 patients developed IPA. In contrast, six of eight patients who became positive for LA during nonneutropenic periods developed IPA. Transient Aspergillus antigenemia was more frequently encountered during neutropenia (2.9%) than during nonneutropenic periods (0.2%). The plasma BDG concentration increased at the nadir of neutropenia in 36 of 45 patients who had no signs of IPA, and it exceeded the level of 20 pg/mL in 2 patients.

CONCLUSIONS. Both BDG and LA have a low sensitivity and a high specificity for IPA. However, the false-positive rate of LA increases during neutropenic periods. Caution should be exercised in interpreting the results of these blood tests, especially when patients are neutropenic. Cancer 1999;86:274–81.

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KEYWORDS: invasive pulmonary aspergillosis, Aspergillus latex agglutination test, (1→3)-β-D-glucan, neutropenia, hematologic malignancy, chemotherapy, galactomannan antigen.
mainly used LA for IPA diagnosis in this study. Because the hepatorenal system rapidly eliminates this antigen from the peripheral blood, the sensitivity of LA varies from 43% to 70%. Although its specificity is as high as 90%, we have frequently encountered false-positive results, especially when the samples were drawn from neutropenic patients.

BDG is a ubiquitous component of fungi, and the determination of its plasma concentration is another useful screening method for deep mycosis, including IPA. Yoshida et al. developed a direct measurement system of plasma BDG concentration using factor G, which is a highly sensitive natural detector of this polysaccharide derived from the horseshoe crab. The sensitivity and specificity for deep mycosis were reported to be 90% and 100%, respectively. However, the clinical utility in the diagnosis of IPA remains to be fully established.

In this study, we evaluated the efficacy of LA and BDG measurement in the diagnosis of IPA and investigated the causes of the false-positive results, especially during neutropenic periods.

PATIENTS AND METHODS
Patients and Conditions of Hospitalization
We prospectively examined 88 consecutive patients with hematologic malignancies for IPA from January to November 1997. At admission, informed consent was obtained from all patients. All patients received either fluconazole (200 mg or 400 mg/day) or itraconazole (200 mg/day) as antifungal prophylaxis. During neutropenia that followed chemotherapy, they were isolated in laminar air-flow-protected rooms. Axillary temperature was measured three times a day. When the first febrile episode occurred, we empirically started both beta lactam antibiotic and aminoglycoside. The intravenous administration of amphotericin B was added when the fever persisted for more than 5–7 days.

Clinical Specimens
From the initiation of chemotherapy until death or discharge, blood samples were drawn weekly and subjected to blood cell counts, LA (Pastorex Aspergillus, Sanofi, Diagnostic Pasteur, Paris, France), the Candida antigen test (Cand-Tec, Ramco, Houston, TX), and the determination of the plasma BDG concentration (Fungi-Tec, Seikagaku Corporation, Tokyo). Neutropenia was defined as a neutrophil count of less than 500/µL. The cutoff level of BDG measurement was 20 pg/mL.

Computed Tomography Scanning Criteria for IPA Diagnosis
We performed computed tomography (CT) scans of the chest if the patients had any signs of pulmonary infection or antibiotics-resistant fever. CT showed either halo signs or air-crescent signs as indicators of IPA. The halo sign is highly indicative of IPA and occurs in the early stage of IPA. We defined these two signs as indicators of IPA.

Criteria for IPA Diagnosis and Candidal Infection
We diagnosed patients as definitely having IPA when they had histologic evidence of tissue invasion by branched septate hyphae of the lung together with a lack of response to antibacterial agents and positive culture for Aspergillus species from the sputa or lung. Those who had the above-mentioned CT signs and persistent fever that was unresponsive to the broad-spectrum antibiotics, but lacked histopathologic evidence, were diagnosed with suspected IPA. Histopathologic evidence meant identification of branched septate hyphae by either autopsy or biopsy. A positive blood culture of Aspergillus was also regarded as histopathologic evidence. Neither smear nor cytology on bronchial lavage, tracheal aspirate, urine, or sputum was included in the histopathologic evidence.

Samples from non-IPA patients were regarded as non-IPA samples. Some samples from IPA patients were included in the non-IPA samples if they were drawn either before the first febrile episode or after the improvement of IPA, fever lysis, and normalization of the chest CT scans with or without scar formation. The other samples from IPA patients were defined as IPA samples.

Patients were considered to have invasive candidiasis if blood cultures were positive for Candida species or if there was histopathologic evidence at biopsy or autopsy.

Criteria and Analysis of Transient Aspergillus Antigenemia
When we encountered positive samples for LA from afebrile patients who had no radiologic or other serologic signs of IPA and did not develop IPA in their clinical course, we considered these samples “transient Aspergillus antigenemia.” If sufficient samples were available, they were further tested by enzyme-linked immunosorbent assay (EIA) for Aspergillus galactomannan antigen or by polymerase chain reaction. In our study, DNA extraction from serum samples and PCR amplification were performed as described previously.

To determine the effect of contamination, sam-
samples from a healthy volunteer, which were deliberately contaminated by the skin, hair, and dust of the laboratory and patients’ rooms, were subjected to LA.

To eliminate the possibility of cross-reactions between the galactomannan antigen and drugs, normal samples were mixed with drugs commonly used for the treatment of hematologic malignancies and were tested for LA. These samples contained amphotericin B (0.005 mg/mL), fluconazole (0.05 mg/mL), ceftadide (0.01 mg/mL), imipenam/cilastin (0.01 mg/mL), amikacin (0.1 mg/mL), tobramycin (0.1 mg/mL), ceftriaxone (0.01 mg/mL), epiphoxodin (0.01 mg/mL), doxorubicin (0.01 mg/mL), daunorubicin (0.01 mg/mL), vincristine (0.01 mg/mL), and famotidine (0.01 mg/mL).

To evaluate the influence of neutropenia per se on the result of LA, 100 samples drawn from nonneutropenic immunocompromised patients at our hospital (with solid tumors, diabetes mellitus, autoimmune disease, and cirrhosis) were also tested for it.

**Statistics**
Data were analyzed for statistical significance by the two-tailed Fisher exact test or by the chi-square test. The prognostic value of LA was assessed with the log rank test. Values of $P < 0.05$ were considered significant.

**RESULTS**

**Patient Characteristics**
We examined 88 consecutive patients with hematologic malignancies. They included acute myeloblastic leukemia ($n = 27$), acute lymphoblastic leukemia ($n = 15$), chronic myelocytic leukemia ($n = 8$), chronic lymphocytic leukemia ($n = 1$), non-Hodgkin lymphoma ($n = 25$), Hodgkin disease ($n = 1$), myelodysplastic syndrome ($n = 5$), and others ($n = 6$). The median age was 50.0 years (range, 16–81 years). Seventeen patients (19.3%) received hematologic stem cell transplantation. Thirty-five patients (39.8%) had progressive hematologic disease.

**Diagnosis of IPA**
Sixteen patients (18.2%) were diagnosed with IPA. Eight cases (50%) were definite IPA and the other 8 (50%) were suspected IPA. Characteristics of these IPA patients are summarized in Table 1. Among these
patients, the duration of the neutropenia was 28.4 ± 20.5 days on average.

Results of LA
We obtained 872 serum samples (124 samples from IPA patients and 748 samples from non-IPA patients). Twenty-eight of the 124 IPA samples (23%) and 16 of the 748 non-IPA samples (2%) were positive for LA. Eleven of the 16 IPA patients (69%) and 8 of the 72 non-IPA patients (11%) became positive for LA during observation. The sensitivity, specificity, positive predictive value, and negative predictive value of LA were 69%, 89%, 58%, and 93%, respectively. Among the eight non-IPA patients who became positive for LA, one was diagnosed as invasive candidiasis, and another who suffered from persistent fever despite the prophylactic administration of antibiotics and amphotericin B did not meet our criteria for IPA. The other six patients satisfied the criteria for the false-positive result of LA.

LA became positive 25.8 ± 18.8 days after the onset of fever, and the CT diagnosis preceded LA by an average of 11.3 ± 19.9 days. Of the 16 IPA patients, there were only 2 for whom LA became positive before the establishment of a diagnosis of suspected IPA.

Samples with Transient Aspergillus Antigenemia
Seven samples from six patients satisfied the criteria for transient Aspergillus antigenemia. The characteristics of these samples are summarized in Table 2. Six of the seven samples were drawn during neutropenic periods. For these patients, the duration of neutropenia was 18.3 ± 13.5 days on average. Three samples tested by EIA were all positive, and Aspergillus specific DNA was identified in two samples (Table 2). Of 204 non-IPA samples drawn during neutropenic periods, 6 samples were determined to be transient Aspergillus antigenemia (2.9%). On the other hand, of 544 non-IPA samples drawn during nonneutropenic periods, only 1 satisfied the criteria of transient Aspergillus antigenemia (0.2%). The transient Aspergillus antigenemia was observed more frequently during neutropenic periods (P = 0.0023). The neutrophil counts of the samples with transient Aspergillus antigenemia and the positive samples from IPA patients are shown in Figure 1. The false-positive results were not correlated with Candida colonization, results of Cand-Tec, or antifungal prophylaxis (data not shown).

None of the 100 samples taken from nonneutropenic immunocompromised hosts were positive for LA. Similarly, all the deliberately contaminated and drug-supplemented samples were also negative for it.

Association between Neutropenia and the Results of LA
LA became positive in 19 patients. Eleven patients (58%) became positive for LA only during the neutropenic periods and 2 patients (18%) developed IPA during their clinical course. In contrast, 6 of the 8 patients whose LA became positive during the nonneutropenic periods developed IPA (75%). This difference was statistically significant, with an odds ratio of 12 (P = 0.0237, 95% confidence interval: 1.29−111) (Table 3). The positive results of LA for nonneutropenic patients were more predictable for the progression to IPA than the results for neutropenic patients.

Results of the Measurement of Plasma BDG Levels
We obtained 885 samples (129 IPA samples and 756 non-IPA samples). Thirty-four of the 129 IPA samples

<table>
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<tr>
<th>No.</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>Disease</th>
<th>State of hematologic malignancy</th>
<th>Neutrophil count (μL)</th>
<th>No. of samples with transient Aspergillus antigenemia/total no. of samples</th>
<th>Levels of EIAa</th>
<th>Result of PCR</th>
<th>Duration of neutropenia</th>
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<tr>
<td>1</td>
<td>F</td>
<td>56</td>
<td>AML</td>
<td>CR</td>
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<td>NHL</td>
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<tr>
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<td>CR</td>
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<td>48</td>
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<td>47.4 ± 24.5</td>
<td>73.4 ± 130.2</td>
<td></td>
<td>18.3 ± 13.5</td>
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</tbody>
</table>

a The cutoff level of EIA for aspergillus galactomannan antigen is 1.0 μg/mL.

and 32 of the 756 non-IPA samples (4%) were positive for BDG assay.

Thirteen of the 16 IPA patients (81%) and 9 of the 72 non-IPA patients (13%) became positive for BDG assay. The sensitivity, specificity, positive predictive value, and negative predictive value were 81%, 88%, 59%, and 95%, respectively. Of the nine non-IPA patients who became positive for BDG assay, three were diagnosed as having invasive candidiasis, four had persistent fever in spite of the combined use of antibiotics and antifungal agents, and the other two had no evidence of fungal infection. Seven samples from the latter two patients were regarded as false-positive. Six of the seven samples (86%) were drawn during neutropenic periods.

BDG assay became positive 25.6 ± 14.4 days after the onset of fever, and the CT diagnosis preceded BDG by an average of 10.3 ± 16.3 days. Of 16 IPA patients, there were only 2 for whom BDG assay became positive before the establishment of a diagnosis of suspected IPA.

Association between Neutropenia and Plasma BDG Concentration
For 45 patients who had no signs of fungal infection during hospitalization, the average levels of plasma BDG were 5.43 ± 4.63 pg/mL before chemotherapy and 8.12 ± 4.90 pg/ml at the nadir of myelosuppression. The plasma BDG concentration increased at the nadir of neutropenia in 36 of 45 patients who had no signs of IPA and exceeded the level of 20 pg/mL in 2 patients (4.4%).

DISCUSSION
It has been reported that LA has a low sensitivity and a high specificity. In patient-based analysis, we demonstrated a sensitivity of 69%, a specificity of 89%, and a positive predictive value of 58%, which were comparable with previous reports.4,10 However, in sample-based analysis, only 24 samples (23%) were positive among the 124 IPA samples. Frequent sample collection is therefore required for patients suspected to have IPA.

BDG assay is a newly developed method of detecting deep mycosis. A large study has been performed to assess the usefulness of this test in the diagnosis of deep mycosis, for which Yoshida et al. reported that the sensitivity and the specificity were as high as 90% and 100%, respectively.7 Our study demonstrated a sensitivity of 81%, a specificity of 88%, and a positive predictive value of 59%, which were comparable to our results for LA. Although BDG assay cannot identify the genus of infected organisms, we can still use it as an adjunctive method for the diagnosis of IPA. However, in sample-based analysis, only 34 samples (26%) were positive for BDG assay among the 129 IPA samples. Thus, frequent BDG determination is also required for IPA diagnosis.

In this study, we spotlighted the false-positivity of results for neutropenic patients, which lowered the results’ positive predictive value. In our study, it might be possible that early empiric amphotericin B administration prevented the progression of IPA. However, of eight patients who became positive for LA but did not develop IPA in their clinical course, six were diagnosed with transient Aspergillus antigenemia and did not receive the empiric administration of amphotericin B. Therefore, the empiric antifungal treatment did not influence our results.
We suspected contamination as a cause of the false-positive results. However, all the deliberately contaminated samples and all the samples from the nonneutropenic immunocompromised patients were negative for LA. For these reasons, the false-positive results would not have been due to artificial contamination. Because only the patients with hematologic malignancies produced false-positive results for LA, we could not neglect the possibility of cross-reaction between the galactomannan antigen and drugs commonly used for the treatment of hematologic malignancies. We evaluated the possible cross-reaction in vitro by mixing the normal sera from healthy volunteers with an excessive amount of anticancer drugs, antibiotics, antifungal drugs, and ranitidine, but all the samples were negative for LA. Although there was a possibility of the cross-reaction occurring between the metabolites of any of these drugs and the galactomannan antigen, we did not find any correlation between the false-positive samples and administration of these drugs (data not shown). Therefore, it seemed unlikely that the false-positive results for LA resulted from the cross-reaction with the drugs or their metabolites.

To investigate the false-positive results for LA, we analyzed our data in two ways. One was serum-based analysis and the other was patient-based analysis. We believe that there were problems in the analysis based only on the numbers of sera because each serum sample from the same patient was not statistically independent. However, we could not categorize the patients as either “neutropenic” or “nonneutropenic” because they had both neutropenic and nonneutropenic periods. In patient-based analysis, eight patients became positive for LA during nonneutropenic periods, and six of these eight patients developed IPA during their clinical course. In contrast, only 2 of the 11 patients who became positive for LA only during neutropenic periods developed IPA (Table 3). This difference was statistically significant (odds ratio 12, 95% confidence interval: 1.29–111). In serum-based analysis, of 204 non-IPA samples drawn during neutropenic periods, 6 samples were diagnosed as transient Aspergillus antigenemia (2.9%). On the other hand, of 544 non-IPA samples drawn during nonneutropenic periods, only one satisfied the criteria of transient Aspergillus antigenemia (0.2%). The transient Aspergillus antigenemia was observed more frequently during neutropenic periods ($P = 0.0023$). Both analyses showed that LA had a lower specificity during neutropenic periods than during nonneutropenic periods.

Although the positive predictive value of LA varies among reports from 56% to 100%, it tended to be low for patients who underwent bone marrow transplantation. Sulahian et al. reported that in 31 of 169 BMT patients (19%) without clinical signs of aspergillosis, Aspergillus antigen detected with EIA was occasionally positive in samples taken within the first month after BMT, giving a specificity of 81% for these patients. Recently, Machetti et al. reported a similar result. They compared the efficacy of LA and EIA in the diagnosis of IPA after bone marrow transplantation and reported that EIA was more sensitive but slightly less specific than LA. However, the positive predictive value of EIA was 50%, which was lower than that of LA (67%) in their study. It is noteworthy that most of the false-positive samples were obtained during the first month after transplantation, although detailed data were not shown in their article. In our study, the positive predictive value of LA was also reduced during neutropenic periods. When these reports are considered, it appears that the antigen detection methods have a low positive predictive value in the evaluation of neutropenic patients. The positive predictive value is determined by the sensitivity, the specificity, and the incidence rate. Because both the sensitivity and the specificity are fixed in each diagnostic test, the positive predictive value of LA is determined by the incidence rate. Owing to the higher incidence rate of IPA during neutropenic periods, the positive predictive value of LA is expected to be higher during neutropenic periods, but our study gave a contrary result. It is likely that the high rate of incidence of the false-positive samples during neutropenic periods lowered the positive predictive value of LA.

Our study suggests that the false-positive results of LA or EIA were not due to either contamination or cross-reaction with drugs or their metabolites, but due to the presence of circulating Aspergillus galactomannan antigen itself. Because of the difficulty of obtaining a blood culture of Aspergillus, it was difficult to demonstrate its presence using conventional blood culture. However, using the antigen detection methods, we demonstrated the presence of the transient Aspergillus antigenemia in neutropenic patients. The elevation of plasma BDG concentration during neutropenia and the PCR results with two samples containing transient Aspergillus antigenemia also support this observation, because these assays recognize different fungal components from those of LA or EIA. We suppose that Aspergillus may enter the bloodstream from the upper respiratory tract or inserted catheter sites and that it may be cleared without the development of IPA in some patients. The clinical significance of transient Aspergillus antigenemia and the portal of Aspergillus in these patients are subjects for further investigation.
In evaluating the usefulness of LA in IPA diagnosis, we must address not only the specificity and sensitivity of these tests, but also whether they provide the diagnosis early enough to impact on clinical decision-making and patient outcomes. For 12 of the 16 IPA patients (87.5%), neither LA nor BDG assay preceded the establishment of IPA diagnosis. CT diagnosis preceded LA by 11.3 ± 19.9 days and BDG by 10.3 ± 16.3 days on average. This suggests that these blood tests may play a limited role in the early diagnosis of IPA and that this test can serve as a confirmation of the diagnosis for a majority of IPA patients. However, there were 2 patients (12.5%) for whom LA preceded the establishment of IPA diagnosis, so we cannot conclude that these blood tests are useless in IPA diagnosis. For these two patients, we encountered an isolated positive result of LA without any specific manifestation of IPA, and we had to differentiate these positive results from the transient Aspergillus antigenemia. Our study showed that positive results of LA indicated the presence of circulating galactomannan antigen itself in a majority of patients, but we cannot say for certain what category of patients with positive LA tests will develop IPA. However, we suspect that the duration of neutropenia may be associated with the development of IPA in patients with positive LA tests. In our study, patients with transient Aspergillus antigenemia had a shorter period of neutropenia (18.3 ± 13.5 days) than patients with IPA (28.4 ± 20.5 days). This may suggest that transient Aspergillus antigenemia occasionally occurs in neutropic patients and that it may lead to the development of IPA in patients with prolonged neutropenia. We suppose that patients with transient Aspergillus antigenemia may have a potential risk of developing IPA.

Our study has two problems that should be discussed. One is its small size. Although we suggest that transient Aspergillus antigenemia occurs in neutropic patients and that LA is less specific during neutropenia, we treated only six patients with transient Aspergillus antigenemia, and this number of patients seems to be too small to allow any definite conclusions. Our study is yet preliminary and awaits further investigation. The other problem is the uncertainty of the diagnosis of IPA. It is usually difficult to make an accurate diagnosis of IPA in all the suspected patients. However, our conclusion is dependent on the accuracy of IPA diagnosis in our 16 IPA patients. In this study, IPA was pathologically identified in eight patients, but for the other eight patients we could not obtain pathologic evidence of IPA. They were diagnosed as suspected IPA using the criteria of IPA diagnosis. However, in many institutions the diagnostic criteria are used for IPA diagnosis and antifungal treatment is initiated based on these diagnostic criteria. Because both the halo and air-crescent signs are highly specific to IPA in neutropenic periods, it might be reasonable to treat patients with an antibiotic-resistant fever and these CT signs as IPA patients, even when IPA is not pathologically identified in these patients. We therefore suppose that our study can provide some information for neutropenic patients with hematologic malignancies.

In conclusion, positive results of LA highly suggest the presence of galactomannan antigen in the blood but do not necessarily predict the progression to IPA, especially in neutropenic patients. Patients who have a short period of neutropenia have a low risk of IPA, even when LA becomes positive in these patients. However, the positive results of LA in patients with prolonged neutropenia may predict progression to IPA. We must consider patients’ neutrophil counts and the duration of neutropenia in interpreting the results of LA.

REFERENCES


