Follow-Up of Anti-*Aspergillus* IgG and IgA Antibodies in Bone Marrow Transplanted Patients With Invasive Aspergillosis

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A total of 89 patients at risk for, or with invasive aspergillosis (IA) were recruited from bone marrow transplantation (BMT) units in two Lisbon hospitals, and followed for 2½ years to monitor their immune response. Of these patients, six developed probable IA, from which five died. The presence of serum IgG or IgA antibodies against seven *Aspergillus* recombinant antigens was assessed in patients with IA, using an enzyme-linked immunosorbent assay (ELISA). In parallel, the serum levels of galactomannan (GM) were

also monitored, using the Platelia *Aspergillus* kit (Sanofi Pasteur, Marnes-la-Coquette, France). Superoxide dismutase (Sod) and 94 kDa were the most immunogenic antigens for IgA, while the IgG pattern of recognition changed from patient to patient. From our results we conclude that although follow-up of antibodies against these antigens should not be used as a diagnostic method, patients with IA do produce an immune response that may influence disease outcome. J. Clin. Lab. Anal. 16:156–162, 2002. ©2002 Wiley-Liss, Inc.

Key words: *Aspergillus*; IgG; IgA; ELISA

INTRODUCTION

Invasive aspergillosis (IA) is a common fungal infection in patients undergoing cytotoxic chemotherapy for hematological malignancies (1). It is a major cause of death at leukemia treatment centers, and bone marrow and solid organ transplantation units (2–5). Despite improvements in the recognition, prophylaxis, and treatment of this disease, it is still associated with high morbidity and mortality (6). The crude mortality rate of IA reaches 100%, resulting, at least partially, from difficulties in obtaining a reliable diagnosis in early phases of the disease, which often leads to a fatal outcome even in the presence of adequate therapy (7–9).

The detection of circulating fungal antigen galactomannan (GM) is a sensitive tool for diagnosing *Aspergillus fumigatus* infection in hematological at-risk patients. Detection using a commercial enzyme-linked immunosorbent assay (ELISA) kit has increased sensitivity (92.6%) and specificity (95.4%) (10). An additional advantage is the possibility of monitoring the antigen titers during treatment (11–13).

In the present study we analyzed a group of 89 bone marrow transplantation (BMT) patients in view of: 1) establishing the prevalence of IA in patients that were bone marrow

transplanted; 2) establishing the relative occurrence of IA depending on the type of transplant (allogeneic or autologous); 3) testing the IgG and IgA responses against a panel of *Aspergillus* recombinant antigens in patients with IA; and 4) verifying the role of these antibodies in disease outcome. The recombinant antigens used were: 18 kDa RNase, 88 and 94 kDa dipeptidylpeptidases, one superoxide dismutase (Sod), one catalase (Cat), one metalloprotease (Mep), and one peptidase (Pep) (all generously supplied by M. Monod, Centre Hospitalier Universitaire Vaudois, Lausanne).

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PATIENTS AND METHODS

Patients

Between October 1997 and July 1999, 89 BMT patients from two transplantation centers in Lisbon—the BMT Unit at the Hospital de Santa Maria (HSM), and the BMT Unit at the Instituto Português de Oncologia (IPO), Lisbon, Portugal—were included in this study. Blood samples were collected from BMT patients with the following frequency: 1) one sample before transplant, 2) two samples per week during hospitalization, and 3) every time the patient came for a follow-up consultation. Serum samples were kept at -20°C until they were used.

IA Definition Criteria

The criteria for classifying IA as definite include the demonstration of *Aspergillus* in pulmonary biopsies or in histopathology obtained by invasive methods. Because of the risk associated with such procedures, they were not performed in these patients. Therefore, we decided to classify our cases as “probable IA” (14), taking into account that all patients had a positive sputum culture for *Aspergillus* species; compatible radiological signs on computed tomography (CT) (such as the “halo sign” or “air crescent sign”); positive detection of serum GM; and symptoms of lower respiratory tract infections, such as cough, pleuritic chest pain, dyspnea, hemoptysis, and pleural rub.

GM Quantification in Serum Samples

Patients were selected to perform this test if they were suspected to have IA. In each patient, GM was only detected when all samples had been collected. If a positive result was obtained, the test was conducted in the remaining samples of such patients. GM-positive patients also had all serum samples tested for IgA and IgG anti-*Aspergillus* recombinant antigens. GM determination was performed using the Platelia *Aspergillus* kit (Sanofi Pasteur, Marnes-la-Coquette, France). The procedures of the technique, including the cut-off value, were in accordance with the manufacturer’s instructions.

Determination of Serum IgA and IgG Optical Densities

IgA and IgG optical densities (ODs) were determined using a solid-phase ELISA. Plates were coated with each of the following recombinant *Aspergillus* antigens: SOD, MEP, CAT,

TABLE 1. Distribution of patients according to the type of transplant in each BMT unit

BMT unit	Autologous BMT	Allogeneic BMT	Total
IPO	2	29	31
HSM	27	31	58
Total	29	60	89

IPO, Instituto Português de Oncologia; HSM, Hospital de Santa Maria.

TABLE 2. Distribution of patients with probable invasive aspergillosis according to the type of transplant in each BMT unit

BMT unit	Total of patients	IA patients/total (%)	IA patients with allogeneic BMT/total (%)	IA patients with autologous BMT/total (%)
IPO	31	2/31 (7%)	2/31 (7%)	0/31
HSM	58	4/58 (7%)	3/58 (5%)	1/58 (2%)
Total	89	6/89 (7%)	5/89 (6%)	1/89 (1%)

IPO, Instituto Português de Oncologia; HSM, Hospital de Santa Maria.

PEP, 88 kDa, 94 kDa, and 18 kDa. Serum samples were diluted 1:500 for IgG and 1:100 for IgA in PBS Tween 0.1% BSA 1%, 100 μL placed in duplicate wells, and incubated 1 hr at 37°C . The supernatants were discarded, and the plates were washed with PBS Tween 0.1%. Anti-IgG (H + L) and anti-IgA conjugated with peroxidase (Sigma, St. Louis, MO) were prepared in PBS Tween 0.1% and BSA 1% at 1:1,000. Then 100 μL of this solution were distributed in each well, and plates were incubated for 1 hr at 37°C . The supernatants were discarded, and the plates were washed with PBS Tween 0.1%. Peroxidase activity was assessed at room temperature using the substrate OPD (1 mg/ml; Sigma) dissolved in citrate buffer (pH 5.2) with H_2O_2 (1 $\mu\text{L}/\text{mL}$); 100 μL of the substrate were distributed in each well. Reactions were stopped by adding of 50 μL of H_2SO_4 10% prior to reading the plates in a automated reader at 492 nm. Negative and positive controls, as well as a cut-off serum, were included in all assays. Samples were considered to be positive if their OD was above the cut-off value.

RESULTS

A total of 89 patients were included in the study (Table 1): 31 were admitted at the BMT Unit at IPO, and 58 at the BMT Unit at HSM. Twenty-nine patients had an autologous BMT, and 60 an allogeneic BMT. Five patients (6%) with probable IA had acquired the infection after an allogeneic transplant; one (1%) had acquired it after an autologous transplant (Table 2). Five of the patients (patients 14, 15, 32, 39, and 53) with probable IA (four allogeneic and one autologous) died; one patient (allogeneic transplant) survived (Table 3).

The IgG response against *Aspergillus* recombinant antigens is shown in Figs. 1 and 2. The 94 kDa antigen appeared

TABLE 3. Patients diagnosed with probable invasive aspergillosis

Patient number	Type of transplant	Positive GM samples	Disease outcome
14	Allogeneic	2	Dead
24	Allogeneic	3	Survived
53	Allogeneic	4	Dead
15	Autologous	1	Dead
32	Allogeneic	5	Dead
39	Allogeneic	1	Dead

to be the most immunogenic. When the samples collected before and after transplant were compared, all patients, with the exception of patient 32, showed an increase in IgG antibody production against this antigen.

Patient 32 did not have any increase in the antibody response, except for the anti-88 kDa IgG, although all of his samples were positive for GM, even before transplant. Patients 39 and 15 died 20 days and 1 month, respectively, after transplant. However, they showed an increase of the antibody anti-94 kDa antigen IgG. If clinical signs of IA were evident, and the first sample of serum was positive for GM, it was seen as a decrease in the antibody titer.

At day 7 after transplant, patient 53 had a significant production of IgG anti-88 kDa, which was high until day 22, 4 days before the clinical signs of IA and GM appeared. IgG anti-94 kDa was also high for a period of 22 days, and decreased when the second sample of serum was positive for GM. There was also an increase in the antibody titers anti-Sod and Cat. After day 57 the antibody against all the recombinant antigens decreased, remaining low until the patient died about 2½ months after transplant.

In relation to patient 14, the most significant immune response was also the IgG anti-94 kDa antigen, which remained high until day 85 after transplant. At that time, the first sample

positive for GM was detected and the patient showed clinical signs of IA; the antibody titer decreased thereafter. This patient died of IA about 4 months after BMT.

The only patient who survived IA was first GM-positive at day 17 after transplant, when a significant antibody response anti-Sod was also shown. Subsequently, all of this patient's samples were negative for GM until day 119, while the IgG levels decreased.

The results of the IgA study are shown in Figs. 3 and 4. IgA levels were low in every patient, for all antigens, with the exception of the one patient who survived IA (patient 24). In this patient a strong IgA response anti-94 kDa antigen was observed, which occurred in parallel with the increase in IgG response anti-Sod, and with the first GM-positive serum sample.

In patient 53 there is a slight increase in IgA anti-94 kDa antigen at day 7 after transplant, and then another at day 43, after which that level was maintained until the last sample. However, this increase was never higher than the levels of serum IgA presented before the transplant.

DISCUSSION

It is estimated that IA occurs in 5–25% of all leukemia patients, in 5–10% after allogeneic BMT, and in 0.5–5% af-

Fig. 1. Longitudinal analysis of serum anti-*Aspergillus* IgG in BMT patients with IA (patients 14, 24, and 53). In the x axis are represented days from transplant, and in the y axis are the OD values of each sample. Cut-off

serum OD values: Mep (0.21), Pep (0.31), 88 kDa (1.4), 18 kDa (1.37), 94 kDa (0.55), Cat (1.1), and Sod (0.22).

Fig. 2. Longitudinal analysis of serum anti-*Aspergillus* IgG in BMT patients with IA (patients 15, 32, and 39). In the x axis are represented days from transplant, and in the y axis are the OD values of each sample. Cut-off

serum OD values: Mep (0.21), Pep (0.31), 88 kDa (1.4), 18 kDa (1.37), 94 kDa (0.55), Cat (1.1), and Sod (0.22).

ter autologous BMT (8,15–19). Similar results were observed in this study, even though all our cases were classified as probable IA: a global incidence of 7% was found (6% in allogeneic and 1% in autologous graft patients).

Mortality in IA-infected patients approaches 100% if the disease is not treated, which occurs primarily because of the inability to establish a correct diagnosis (7–9). In this study we observed a global mortality of 83% (5/6). The occurrence of IA had a bimodal distribution: patients 15, 32, and 39 developed the disease during the first weeks after transplant, whereas in patients 14, 24, and 53 the disease was observed months after transplantation. Previous studies also showed that IA occurred in a bimodal distribution, 2–3 weeks or 2–3 months after transplantation (20–22).

IA is a very difficult syndrome to diagnose, since, as previously stated, definitive proof implies the demonstration of the fungus in tissue specimens together with a positive culture (23), obtained by invasive procedures. However, these are not recommended in IA-suspected patients because of their extremely debilitated physical condition (10). As a consequence, clinicians have to combine clinical, radiological, and, when available, microbiological criteria, which often lack specificity and sensitivity, and frequently require a high fungal burden (23).

The detection of a circulating fungal antigen such as GM, a major *Aspergillus* exoantigen released during invasive disease, is currently considered to be an adequate indirect method for diagnosing IA (11,24–27). In this study, GM-positive samples were used as one of the criteria to diagnose probable IA.

The aim of this study was to verify whether the IA patients submitted for BMT were able to mount an antibody response against *Aspergillus* recombinant antigens, since it was considered that because of the severely immunosuppressed condition of these patients they were unable to mount an appropriate antibody response against *Aspergillus* (28–33). Overall, we observed that the 94 kDa antigen was the most immunogenic in terms of IgG response, which to us was not completely surprising as this antigen is a dipeptidylpeptidase (34), is probably involved in the promotion of lung matrix colonization (35), and is available in the lung milieu. Patient 53 had the most significant IgG production against the 88 kDa antigen, another dipeptidylpeptidase (36). However, there were other IgGs that reached the same levels they had before the transplant, such as the IgG anti-94 kDa and Cat. Patient 24 had the stronger IgG response anti-Sod, which occurred in parallel with the increase in IgA anti-94 kDa antigen, and with the first GM-positive serum sample. The fact that this

Fig. 3. Longitudinal analysis of serum anti-*Aspergillus* IgA in BMT patients with IA (patients 14, 24, and 53). In the x axis are represented days from transplant, and in the y axis are the OD values of each sample. Cut-off

serum OD values: Mep (0.17), Pep (0.19), 88 kDa (0.35), 18 kDa (0.2), 94 kDa (0.42), Cat (0.26), and Sod (0.81).

was the only patient who survived, and that by the time the first serum sample turned out to be positive for GM a strong IgG and IgA response anti-94 kDa Cat and Sod *Aspergillus* antigens also occurred, is in accordance with findings by Latgé (35). He suggested that an increase in antibody titer at the end of immunosuppression is indicative of recovery from IA. On the other hand, absence of antibody titer, or declining antibody levels suggest a poor prognosis. We also verified that every patient who succumbed to IA either did not have any antibody response (patients 15, 32, and 39) or their level declined as IA diagnostic criteria appeared.

It was clear to us that patients who had IA months after transplant were able to develop an immune response towards *Aspergillus*, distinct from the one presented before transplant, while those who developed the disease shortly (weeks) after the transplantation were not able to develop such a response—certainly as a consequence of the severe immunosuppression still present at that time. These results seem to contradict, at least in relation to patients who developed IA several months after transplant, those authors who state that anti-*Aspergillus* antibodies detected in severely immunosuppressed patients probably are not a result of invasive infection, but more likely represent immunoglobulins formed before the immunosup-

pression (16,29,30,31,33). Furthermore, some patients (patients 14, 24, and 53) had higher IgG antibody levels after transplantation than before transplantation.

In view of the results presented here, it appears that some patients do mount an anti-*Aspergillus* antibody response, and that these antibodies may influence disease outcome. However, these results should be further explored.

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Fig. 4. Longitudinal analysis of serum anti-*Aspergillus* IgA in BMT patients with IA (patients 15, 32, and 39). In the x axis are represented days from transplant, and in the y axis are the OD values of each sample. Cut-off

serum OD values: Mep (0.17), Pep (0.19), 88 kDa (0.35), 18 kDa (0.2), 94 kDa (0.42), Cat (0.26), and Sod (0.81).

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