Aspergillus antigen testing in bone marrow transplant recipients

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Abstract

***Aims*—To assess the clinical usefulness of a commercial aspergillus antigen enzyme linked immunosorbent assay (ELISA) in the diagnosis of invasive aspergillosis (IA) in bone marrow transplant recipients, and to compare it with a commercial latex agglutination (LA) test.**

***Methods*—In total, 2026 serum samples from 104 bone marrow transplant recipients were tested. These comprised 67 sera from seven patients who had died with confirmed IA, 268 sera from nine patients who had died with suspected IA, and 1691 sera from 88 patients with no clinical, radiological, or microbiological signs of IA.**

***Results*—The ELISA was more sensitive than the LA test. All patients who were ELISA positive were also LA positive, and a positive LA result never preceded a positive ELISA. Twelve of 16 patients with confirmed or suspected IA were ELISA positive on two or more occasions, compared with 10 of 15 who were LA positive. ELISA was positive before LA in five patients (range, 2–14 days), and became positive on the same day in the remainder. Aspergillus antigen was detected by ELISA a median of 15 days before death (range, 4–233). Clinical and/or radiological evidence of IA was noted in all patients, and a positive ELISA was never the sole criterion for introduction of antifungal treatment. Two samples (one from each of two patients without IA) gave false positive results.**

***Conclusions*—The aspergillus ELISA is a specific indicator of invasive aspergillosis if the criterion of two positive samples is required to confirm the diagnosis. However, the test is insufficiently sensitive to diagnose aspergillosis before other symptoms or signs are apparent, and hence is unlikely to lead to earlier initiation of antifungal treatment. It is therefore unsuitable for screening of asymptomatic patients at risk of invasive aspergillosis, but does have a useful role in confirming the diagnosis in symptomatic patients.**

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Invasive aspergillosis (IA) is a common life threatening complication of allogeneic bone marrow transplantation (BMT),[1] particularly in patients receiving grafts from unrelated donors.[2] Patients at greatest risk of developing IA are those with delayed engraftment, and those with severe acute or chronic graft versus host disease (GVHD).[1] In BMT recipients, IA is usually relentlessly progressive, with a mortality rate of more than 90% despite treatment.[3] There is some evidence that the mortality rate can be lowered if an early diagnosis of IA can be made, and specific antifungal treatment given.[3–5] This is difficult because of the absence of specific symptoms and because cultures of sputum and bronchoalveolar lavage (BAL) are seldom positive.[6 7] At present, the most reliable method for diagnosing IA is histological demonstration of tissue invasion by fungal hyphae combined with a positive culture for aspergillus. However, biopsies can seldom be obtained from profoundly immunocompromised patients because most are also severely thrombocytopenic or too unwell.

Serological tests for aspergillus antibodies are seldom positive in immunocompromised patients and attention has therefore concentrated on the development of methods to detect antigens of *Aspergillus* spp in body fluids.[8–13] Most effort has been concentrated on the detection of galactomannan (GM), a major cell wall component of these fungi,[14 15] and several commercial tests have been devised. The first of these, a latex agglutination (LA) test (Pastorex Aspergillus, Sanofi Diagnostics Pasteur, Paris, France) uses a rat IgM monoclonal antibody EB-A2 to detect *Aspergillus fumigatus* GM.[16] This test can detect 10–15 ng of GM/ml of serum. Previous evaluations of the LA test in patients with neutropenia have given variable results, with sensitivities ranging from less than 30% to 95%.[17–20] Most studies have found the LA test to have a specificity of 90–100%.[18–21] However, the test has been found to give positive results only during the later stages of the infection.[21 22]

More recently, a commercial test has been developed using a double direct sandwich enzyme linked immunosorbent assay (ELISA) (Platelia Aspergillus, Sanofi Diagnostics Pasteur). This test also uses the rat monoclonal antibody EB-A2 to detect *A fumigatus* GM, but it has a 10 times lower limit of detection than the LA test.[23 24] It has a sensitivity of 67–100% and a specificity of 81–99% when performed with serum samples from neutropenic patients receiving treatment for haematological malignancies.[19 24–26] Previous evaluations of the ELISA have suggested that it might become positive at an early stage of infection in these patients, and GM has been detected in some neutropenic patients before symptoms and signs consistent with IA had become

Table 1 Antigen test results in seven patients with proven invasive aspergillosis (group I)

Patient no.	*Time of first sample*	*No. of samples tested*	*No. of samples positive*		*Time of first positive sample*		*Outcome*	*Comment*
			ELISA	*LA*	*ELISA*	*LA*		
1	D11	12	8	6	D5	D7	Died D20	PM: *Aspergillus flavus*
2	D7	14	4	1	D21	D28	Died D31	PM: *A. fumigatus* and *A. flavus*
3	D4	7	0	0	–	–	Died D25	PM: histology positive
4	D0	4	3	3	D4	D4	Died D8	PM: *A. fumigatus*
5*	–	6	4	3	D18	D21	Died D32	PM: *A. fumigatus*
6	D334	10	2	2	D356	D356	Died D360	Sputum: *A terreus*
7	D640	14	8	5	D651	D665	Died D679	PM: *A fumigatus*

Times refer to the number of days (D) before or after transplant.
Aspergillosis was disseminated in all patients except patient 3 (lung only).
*Patient conditioned but not transplanted; dates taken from start of antifungal treatment.
ELISA, enzyme linked immunosorbent assay; LA, latex agglutination; PM, postmortem examination.

apparent.[25] However, false positive ELISA results have been reported to occur in some patients after BMT.[19] To assess further the clinical usefulness of the aspergillus antigen ELISA and compare it with the LA test, we obtained serum samples from 104 BMT recipients, including 16 with confirmed or suspected IA. The serological test results for this group were correlated with the clinical, radiological, and microbiological findings.

Methods

PATIENTS

Serum samples tested in this investigation were obtained from three groups of patients (n = 104) undergoing bone marrow transplantation at United Bristol Healthcare Trust, more than half of whom received grafts from unrelated donors. Most patients (69%) were children (age ⩽ 17 years); ages of patients ranged from 3 months to 56 years, with a median of 12 years 5 months. The first group of 67 sera was obtained from seven patients who had died with confirmed IA (group I). All these individuals had histological evidence of disease, or had positive microscopy with branching septate hyphae seen in conjunction with positive culture of a respiratory tract or tissue sample. The second group of 268 sera was collected from nine patients who had died with suspected IA (group II). These individuals had two or more of the following features: new infiltrates on chest computed tomography (CT) scans or radiographs, positive bronchoalveolar lavage (BAL) culture, or respiratory symptoms including severe pleuritic chest pain. The third group consisted of 1691 sera from 88 patients with no clinical, radiological, or microbiological signs consistent with a diagnosis of IA (group III). All patients admitted for transplantation or management of late complications received antifungal prophylaxis with oral itraconazole capsules 2.5 mg/kg, replaced with intravenous amphotericin 0.5–1 mg/kg on alternate days when oral medication was not tolerated. The protocol for management of neutropenic fever was treatment with broad spectrum intravenous antibiotics, with the addition of intravenous amphotericin after 72 hours of refractory fever. Ninety eight patients, who were consecutive admissions to the unit over the 18 month period from November 1996 to April 1998, were evaluated prospectively. The LA test had been used prospectively on the unit before the introduction of the ELISA. All patients (n = 6) who were antigen positive by LA in the period March 1995 to October 1996 were re-evaluated by ELISA (patients 2, 4, and 5 in table 1 and patients 1, 3, and 5 in table 2). Clinical data including use of antifungal treatment and categorisation of patients into confirmed, suspected, or unlikely categories of invasive aspergillosis were assessed retrospectively, without reference to aspergillus antigen results.

ANTIGEN TESTING METHODS

Serum samples for prospective analysis were collected twice weekly and tested on the day of collection. Positive samples were frozen overnight and retested the next day. If the result of retesting was positive then the result was telephoned through to the requesting clinician. Sera from six patents evaluated initially by LA were collected twice weekly and tested after being frozen. These samples were tested retrospectively by ELISA after a maximum of two years storage at −20°C. Freezing does not

Table 2 Antigen test results in nine patients with suspected aspergillosis (group II)

Patient no.	*Time of first sample*	*No. of samples tested*	*No. of samples positive*		*Time of first positive sample*		*Outcome*	*Comment*
			ELISA	*LA*	*ELISA*	*LA*		
1	D12	12	4	4	D26	D26	Died D49	No isolates
2	D6	16	3	0	D3	–	Died D88	No isolates
3	D4	67	38	33	D12	D12	Died D245	Sputum: *Aspergillus fumigatus*
4	D1	37	2	ND	D171	–	Died D177	No isolates
5	D3	3	3	3	D3	D3	Died D13	No isolates
6	D3	39	0	0	–	–	Died D125	BAL: *A. fumigatus*
7	D24	63	0	0	–	–	Died D245	No isolates
8	D681	18	7	3	D717	D728	Died D740	No isolates
9	D739	13	0	0	–	–	Died D800	BAL: *A flavus*

Time refers to number of days (D) before or after transplant.
ELISA, enzyme linked immunosorbent assay; LA, latex agglutination; ND, not done; BAL, bronchoalveolar lavage.

Table 3 Clinical and laboratory findings in 16 patients with confirmed or suspected aspergillosis (groups I and II)

Clinical group	Finding	No. of evaluable patients	No. (%) of evaluable patients positive	Time of first positive finding	
				Median	Range
Confirmed IA (n = 7)	Fever	7	6 (86)	25	8–52
	CT	1	1 (100)	10	–
	CXR	7	7 (100)	12	4–52
	ELISA	7	6 (86)	13	4–28
	Culture*	7	1 (14)	8	–
Suspected IA (n = 9)	Fever	9	5 (56)	62	9–176
	CT	4	4 (100)	31	9–56
	CXR	9	8 (89)	31	5–176
	ELISA	9	6 (67)	23	6–233
	Culture*	9	2 (22)	52	49–56

Time of first positive finding refers to number of days before death.
*Antemortem samples.
IA, invasive aspergillosis; CT, computerised tomographic scanning; CXR, chest radiograph; ELISA, enzyme linked immunosorbent assay.

affect detection of GM by ELISA (ECM Williamson *et al*, unpublished observations, 1996). The aspergillus antigen LA and ELISA tests were performed according to the manufacturers' instructions, with the modification that the boiling time of serum with treatment solution was extended from three minutes to 15 minutes, to inactivate blood borne viruses. This modification did not affect ELISA readings (ECM Williamson *et al*, unpublished observations, 1996). Before testing, 300 µl of serum was mixed with 100 µl of treatment solution containing EDTA and boiled for 15 minutes to dissociate immune complexes. The sample was then centrifuged for 10 minutes at 10 000 ×*g*. For the LA test, 40 µl of the supernatant was mixed with 10 µl of sensitised latex reagent on a clean black slide, then rocked for five minutes. Agglutination was recorded as being present or absent.

For the ELISA, 50 µl of supernatant was mixed with 50 µl of horseradish peroxidase conjugated EB-A2 and placed in the wells of a microtitre plate coated with anti-EB-A2 monoclonal antibody. After incubation at 37°C for 90 minutes, the plate was washed five times and 200 µl of chromogen substrate solution, containing tetramethylbenzidine and dimethyl sulphoxide, was added to the wells. The plate was incubated for 30 minutes in darkness before the reaction was stopped with 100 µl of 1.5 M sulphuric acid. The optical density was read at 450 nm and 620 nm. Absorbance ratios were calculated according to the manufacturer's instructions, with all ratios of greater than one being classed as positive. All runs included strong positive, weak positive (tested in duplicate), and negative control sera. For both LA and ELISA tests, a sample was only considered positive if a positive result was obtained on retesting.

Results

Aspergillus antigen tests were performed on 1691 serum samples from 88 patients with no signs of invasive fungal infection (group III). Two samples (one from each of two patients) gave positive ELISA results. None of these 88 patients later developed IA. Antigen tests were performed on 335 serum samples from 16 patients with confirmed or suspected IA (groups I and II) (tables 1 and 2). All patients had pulmonary involvement; there were no cases of invasive fungal sinusitis. Seven of these patients developed IA as a late complication of transplantation (> 100 days after transplant), and only one of these late cases was neutropenic when IA developed.

Table 1 summarises the antigen test results for seven patients who died with confirmed IA (group I), five of whom died during their initial admission for transplantation and two of whom were readmitted at > 300 days for management of GVHD. In six cases both tests gave positive results, but 29 of the 60 samples from these patients gave positive results by ELISA compared with 20 samples tested by LA. Table 2 summarises the results of the two antigen detection methods in nine patients who had died with suspected IA (group II). None of these patients underwent postmortem examination. In six of these cases, the patient died more than 100 days after transplantation. Six of the nine patients gave positive results by ELISA, compared with four of eight by LA. In the four patients in whom both tests gave positive results, 52 of the 100 samples gave positive results by ELISA compared with 43 samples by LA.

In an attempt to assess the clinical usefulness of aspergillus antigen detection by ELISA, we evaluated the clinical records of the 16 BMT recipients with confirmed or suspected IA (groups I and II). Table 3 summarises the clinical, radiological, microbiological, and serological findings in these patients. In the seven patients with confirmed IA (group I), antigen was first detected a median of 13 days before death. Among the other findings in this group, chest radiological changes were noted in all seven patients a median of 12 days before death, but culture was positive in only one patient (eight days before death). In the nine cases of suspected IA (group II), antigen was detected in six patients, a median of 23 days before death, but culture was only positive in two patients, a median of 52 days before death. Chest radiological changes were detected in eight of these cases, a median of 31 days before death. CT scans were only performed in four of these nine patients, but changes were noted in all four cases, a median of 31 days before death. The reason for commencing antifungal treatment was assessed for all patients with confirmed or suspected IA who became ELISA positive (n = 12). Factors contributing to the introduction of treatment were neutropenic fever in five patients, respiratory symptoms in four, pulmonary radiological changes in five, and a positive antigen test in five. A positive ELISA was never the sole reason for institution of antifungal treatment. Two of six retrospectively evaluated patients were ELISA positive before becoming LA positive. Antigen testing did not contribute to the introduction of antifungal treatment in these patients.

Discussion

Invasive aspergillosis is an increasingly common complication of BMT. Patients who develop chronic GVHD are at particularly high

risk, and these patients represent a major diagnostic challenge because they are often not neutropenic and steroids used to treat GVHD may diminish the febrile response to an infectious process. The response of BMT recipients to antifungal treatment is poor, and death is common once aspergillus infection is established.[3] Early treatment may be associated with a better outcome,[3–5] but early diagnosis is difficult to accomplish. Non-specific clinical and radiological findings, failure to culture the organism, and the lack of a detectable antibody response to infection have led to interest in the development of tests to detect aspergillus antigens in body fluids.[8–15 17–26] Aspergillus GM, a major cell wall component, has been found to circulate in the blood of neutropenic patients with IA. However, concentrations fluctuate during the course of the infection because of antigen clearance by the Kupffer cells of the liver, and it has therefore been suggested that frequent sampling (at least weekly) of patients at risk of IA is necessary.[14 15 17–23 25] This was the strategy used on our bone marrow transplant unit. All patients on the unit were receiving antifungal prophylaxis with either itraconazole capsules or alternate day amphotericin B, which was switched to empirical amphotericin B treatment in the presence of a neutropenic fever of 72 hours duration unresponsive to broad spectrum antibiotics. The effect that the administration of antifungal drugs might have on the release of GM antigen and consequent performance of the test is not known. However, such practice is common in many BMT units and would therefore reflect common clinical application of the test.

Our results are in agreement with those of others,[19 24] who have demonstrated that the ELISA is more sensitive than the LA test for aspergillus antigen, leading to both an increased number of patients being found to be antigen positive, and earlier diagnosis in some patients. However, the ELISA does not appear to become positive sufficiently early to alter clinical management. Assessment of the clinical usefulness of a diagnostic test requires information on the timing of positive test results in relation to the onset of the disease, and information on whether the test alters management. The onset date of invasive aspergillosis is often difficult or impossible to define in a BMT recipient, because the initial symptoms and clinical signs of infection, such as cough and fever, are non-specific. There is an association between invasive fungal infection and viral infections, many of which present with pulmonary involvement, in BMT recipients,[2] which further complicates diagnosis. Therefore, we have chosen to assess whether the ELISA alters clinical management because the dates of administration of antifungal drugs were obtainable for all patients. Although a positive ELISA was a factor in the introduction of antifungal treatment in five patients, it was never the sole reason for starting treatment. Furthermore, ELISA did not contribute to the management of most (11 of 16) patients with IA, either because ELISA was never positive (four of 16), or because a diagnosis had already been obtained by other means (seven of 16 patients).

Other strategies have been evaluated as an adjunct to diagnosing aspergillosis. Thoracic CT scans are more sensitive than plain chest radiographs, and might be useful in some patients[7 27 28] if small nodules and/or small pleural based lesions with a surrounding low attenuation area, the "halo sign", are present. Our study does not provide comprehensive data on the usefulness of CT in BMT recipients, because CT was usually only performed to provide further information on suspicious plain radiographs. Therefore, our data will tend to underestimate the usefulness of this procedure. However, CT scanning might not be achievable in all patients, because of problems of moving very unwell patients and of sedating young children.

Two patients with no other signs of IA gave positive ELISA results but only on one occasion. Advice contained in the ELISA kit suggests that tests should be repeated on a fresh specimen to confirm positive results so these patients would not meet that criterion and cannot be regarded as confirmed false positives.

In conclusion, although the Platelia aspergillus ELISA appears to be a relatively insensitive procedure, it is specific, non-invasive and, unlike CT scanning, can be repeated at frequent intervals and without removing patients from protective isolation. It is insufficiently sensitive to be used for routine screening of asymptomatic patients considered at risk of invasive aspergillosis, but forms a valuable adjunct to diagnosis if there is clinical suspicion that invasive aspergillosis has developed. Because this test is insensitive, the negative predictive value is low; therefore, a negative result would not be an indication for withholding antifungal treatment. Our bone marrow transplantation unit has therefore abandoned twice weekly screening of patients, but the ELISA is still used if there is clinical suspicion of invasive aspergillosis. A combination of strategies is needed to diagnose IA. The combination of radiology and aspergillus ELISA might be useful, and possibly the polymerase chain reaction (PCR) will also have a role in diagnosis, although data comparing PCR and antigen testing are limited, because most studies have looked at late cases, positive by both methods.[29] One retrospective study found PCR to be less sensitive than ELISA,[30] and concluded that neither test anticipated the introduction of antifungal treatment. No current diagnostic test obviates the need for empirical antifungal treatment in BMT recipients.

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