Aspergillus antigen testing in bone marrow transplant recipients

E C M Williamson, D A Oliver, E M Johnson, A B M Foot, D I Marks, D W Warnock

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.


Keywords: invasive aspergillosis; aspergillus antigen; Platelia enzyme linked immunosorbent assay

Invasive aspergillosis (IA) is a common life threatening complication of allogeneic bone marrow transplantation (BMT), particularly in patients receiving grafts from unrelated donors. Patients at greatest risk of developing IA are those with delayed engraftment, and those with severe acute or chronic graft versus host disease (GVHD). In BMT recipients, IA is usually relentlessly progressive, with a mortality rate of more than 90% despite treatment. 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. This is difficult because of the absence of specific symptoms and because cultures of sputum and bronchoalveolar lavage (BAL) are seldom positive. 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. Most effort has been concentrated on the detection of galactomannan (GM), a major cell wall component of these fungi, 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. 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%. Most studies have found the LA test to have a specificity of 90–100%. However, the test has been found to give positive results only during the later stages of the infection. 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. 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. 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
Antigen testing for invasive aspergillosis

ELISA, enzyme linked immunosorbent assay; LA, latex agglutination; ND, not done; BAL, bronchoalveolar lavage.

Time refers to number of days (D) before or after transplant.

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

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Time of first sample</th>
<th>No. of samples tested</th>
<th>No. of samples positive</th>
<th>Time of first positive sample</th>
<th>Outcome</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D11</td>
<td>12</td>
<td>8</td>
<td>6</td>
<td>D5</td>
<td>D7</td>
</tr>
<tr>
<td>2</td>
<td>D7</td>
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<td>D21</td>
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<td>D21</td>
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<td>10</td>
<td>2</td>
<td>2</td>
<td>D356</td>
<td>D356</td>
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<tr>
<td>7</td>
<td>D640</td>
<td>14</td>
<td>8</td>
<td>5</td>
<td>D651</td>
<td>D665</td>
</tr>
</tbody>
</table>

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.

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 patients 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 appear to affect antigen activity.

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

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Time of first sample</th>
<th>No. of samples tested</th>
<th>No. of samples positive</th>
<th>Time of first positive sample</th>
<th>Outcome</th>
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<td>D26</td>
<td>D26</td>
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</tr>
</tbody>
</table>

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)

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

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.

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 aspergillosis infection is established. Early treatment may be associated with a better outcome, 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. 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. 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, who have demonstrated that the ELISA is more sensitive than the LA test for aspergillus antigens, 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, 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 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 sufficiently 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. One retrospective study found PCR to be less sensitive than ELISA, 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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Bones, groans, moans . . . and salivary stones?

A 46 year old man was referred to hospital by his general practitioner with abnormal bone biochemistry. He had presented with poor appetite, fatigue, myalgia, and backache. Serum calcium, corrected for albumin, was 2.63 mmol/litre (reference range, 2.12−2.62), serum phosphate was 0.85 mmol/litre (normal range, 0.7−1.4), and alkaline phosphatase was 367 IU/litre (normal range, 80−280). There was no history of previous fractures or of renal calculi. The parathy- Moreno concentration was raised at 19 pmol/litre (normal range, 1.3−7.5) and the urinary calcium to creatinine ratio was 0.375 (normal range, 0.085−0.65). Bone densitometry pro- vided normal evidence of osteoporosis (T score, −3.05). Ultrasound of his neck revealed a solid lesion of low echodensity at the lower pole of the right lobe of the thyroid gland, typical of a parathyroid adenoma. At surgery the lower right parathyroid gland was excised, and confirmed by histology to be an ad- enoma.

At outpatients one week previous elective parathyroidectomy, the patient reported that he had passed a stone from a salivary gland. He had attended hospital as an emergency two months previously and had been diag- nosed as having sialadenitis of the left submandibular gland with a small calculus present in it. Subsequently, he became exasperated with the pain and manually forced the calculus out of the duct. There was no history of chronic infection or of other pathology to explain the presence of the calculus. The calculus weighed 2 mg and consisted of calcium phosphate (59%) and calcium oxalate (41%).

Sialolithiasis has been reported in hyper- parathyroidism secondary to chronic renal failure but not previously in primary hyperparathyroidism. Salivary stone formation may be promoted by the combined effects of hypercalcaemia and secretory stimulation; the mechanism involves excessive calcium release into the acinar lumina resulting in calcium phosphate aggregates. Such calcium phosphate intermediates may transform into more stable hard deposits. Their saturation in solution varies widely, partly because of the variation in pH that occurs in saliva. As a result, some of these calcium phosphate aggregates may precipitate.

Why do salivary stones occur so much less frequently than urinary stones in hyperpara- thyroidism? Some ions in saliva and urine, such as citrate, inhibit the growth of precipi- tated crystals, whereas others, like calcium and phosphate, accelerate growth. The bal- ance of these and other molecules might favour stone formation in urine but not in saliva. Certainly, mechanisms involved to explain urolithiasis in hyperparathyroidism include hypercalcemia, hyperphosphaturia, and hypocitraturia. However, salivary con- centrations of calcium and phosphate are also raised in primary hyperparathyroidism; the comparative rarity of salivary stones in hyper- parathyroidism probably owes more to spe- cific salivary proteins that control mineralisation, such as statherin and proline rich protein. It is possible that when salivary stones develop in hyperparathyroidism, they arise via the mechanism outlined above, with alterations in the concentrations of calcium and phosphate playing a primary pathoge- netic role. Such a putative similarity in the pathogenesis of primary hyperparathyroidism and nephrolithiasis would be consistent with the observed association between the two conditions. In one large series, six of 56 patients with sialo- lithiasis were reported to suffer from nephrolithiasis as well.

It is impossible to estimate accurately the true extent of any putative link between hyperparathyroidism and sialolithiasis, prec- isely because such a link has been widely recognised. Certainly, most patients with salivary stones are not investigated for abnor- mal bone biochemistry. The time honoured mnemonic has it that hyperparathyroidism and other hypercalcemic states were classi- cally associated with “bones, stones, abdomi- nal moans, and psychic groans.” Although this full blown clinical presentation is rarely seen today, we suggest that it may include salivary stones as well as urinary stones.

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Fatal legionellosis in association with chronic lymphocytic leukemia

Treatment of chronic lymphocytic leukaemia (CLL) with nucleoside analogues may cause T cell dysfunction, thereby predisposing to opportunist infections in addition to bacterial infections as a result of neutropenia and humoral immune dysfunction.1 Cases of Legionella pneumonia as a result of neutropenia and humoral immune dysfunction.2 The following case provides an example of fatal legionellosis in association with chronic lymphocytic leukemia.

A 62 year old male non-smoker had obtained a good partial response after completing four courses of fludarabine treatment for relapse of stage B CLL. He had been treated at diagnosis 2½ years ago with chlorambucil and purinobin but had never received corticosteroids. His general health had been good and he had continued in full time employment throughout. He developed “flu-like” symptoms just before returning to the UK from holiday in Spain and was prescribed co-amoxiclav by his general prac- titioner immediately on arrival. The next day he was admitted to hospital under a general medical team with lobar pneumonia and commenced treatment with ceftazidime. Clarithromycin, ciprofloxacin, and rifampicin were added soon after Legionella pneumophila was suspected (and later confirmed) to be the casual organism, but he died two days later.

There are approximately 200 cases of legionnaire’s disease notified each year to the National Surveillance Scheme in England and Wales, of which half are associated with overseas travel, mainly to Spain or Greece. Immunosuppression, usually from cortico- steroids or human immunodeficiency virus (HIV) infection, is known to predispose to infection and to increase mortality, as is

chronic pulmonary disease. Of the haematological diseases, the risk of legionella seems highest in hairy cell leukaemia, possibly because of impairment of monocyte function, and has been seen after treatment with 2-chloro-2-deoxyadenosine. Opportunistic infections after treatment with fludarabine are usually seen with advanced Rai stage, severe neutropenia, impaired renal function, or concomitant prednisolone treatment. Legionella is uncommon in CLL, although it has been described after treatment with fludarabine.

Treatment with co-trimoxazole is recommended for prophylaxis against pneumocystis in patients receiving nucleoside analogues but is not nucleoside free from its use in HIV infected patients whether this decreases the risk of legionella infection. With the increasing use of fludarabine as a first line treatment, the carrying of an alert card specifying infective and transfusion risks seems warranted.

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Is it useful to test for antibodies to extractable nuclear antigens in the presence of a negative antinuclear antibody on Hep-2 cells?

Antinuclear antibody (ANA) negative lupus has long been recognised as a distinct entity affecting a small number of patients with systemic lupus erythematosus (SLE). Initial estimates of the prevalence of this entity (5% of patients with lupus) were based upon studies using rodent tissues as substrate for antinuclear antibody testing. The increasing use of human epithelial cell lines (Hep-2 cells), which have greater sensitivity for extractable nuclear antibodies (ENA), has meant that new patients with true ANA negative lupus are now rarely encountered.

Many immunology laboratories are faced with a substantial number of requests for antibodies to ENA and double stranded DNA, even in patients with negative ANA, on the grounds that patients with ANA negative lupus might go undetected. Using Hep-2 cells, we have attempted to define the prevalence of ANA negative, anti-ENA positive disease in a series of consecutive, unselected serum samples.

Over a 12 month period, all laboratory requests for antibodies to ENA (antibodies to Sm, Ro, La, and ribonuclear protein) were scrutinised to determine the number of samples that had antibodies to ENA despite a negative ANA on Hep-2 cells. The notes of patients who were ANA negative, anti-ENA positive were examined to verify the clinical diagnosis.

During the 12 month study period, 7077 Hep-2 ANA samples were processed and 468 patients had an anti-ENA profile performed despite an ANA negative status. Of these 468 patients, nine were identified who were ANA negative, anti-ENA positive. Review of their clinical notes indicated that six of these nine patients had previously been ANA positive and were known to have lupus but were receiving immunosuppressive treatment. Only three patients were persistently ANA negative despite positive anti-Ro antibodies before treatment. Thus, the prevalence of anti-ENA positivity combined with a negative ANA was three out of 468 (0.64%). Because ANA negative lupus characteristically presents with cutaneous disease the clinical notes of 90 of the dermatology patients were reviewed. Seven of these nine patients had confirmed lupus erythematosus. Only one patient from the dermatology group had ANA negative, anti-Ro positive lupus before the commencement of immunosuppressive treatment.

Our finding of a low prevalence of anti-ENA positivity in the presence of a negative ANA on Hep-2 cells is in keeping with other studies in the literature. Manousakis et al found that only 0.4% of 243 Hep-2 negative patients with systemic autoimmune disease had positive ANA antibodies and Homburger, reporting on the experience of the Mayo Clinic immunopathology laboratory, stated that anti-ENA antibodies were unlikely to be positive in the presence of a negative ANA result on Hep-2 cells. However, neither of these studies included a clinical evaluation of the ANA negative, anti-ENA positive patients.

We recognise the clinical relevance is subject to potential sources of bias. The failure to scrutinise patients’ notes on all ANA negative samples irrespective of anti-ENA antibody status might have resulted in some patients with strong clinical evidence of connective tissue disease being overlooked. We think it unlikely that this would have greatly changed our findings given the rarity of uniformly seronegative lupus (ANA negative, anti-ENA negative, and anti-DNA negative) and the general acceptance that a repeatedly negative ANA effectively excludes systemic lupus. Second, if clinicians failed to request ENA along with ANA, it is possible that some cases of ANA negative, ENA positive disease would be missed.

Based on these findings and others in the literature we have modified our testing strategy for antibodies to ENA. All requests for anti-ENA antibodies are “gated” by performing an initial ANA screen on Hep-2 cells. Samples that are ANA negative do not proceed to further testing unless there are compelling clinical reasons to suggest lupus.

In conjunction with clinical liaison this testing strategy allows streamlining in busy clinical laboratories.

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Handling of renal biopsies: different approaches reflect a lack of evidence for what constitutes “best practice”

We read ACP Best Practice No 160 “Renal biopsy specimens” with interest. Dr Furness rightly avoids providing a list of specific procedures to follow because, as he points out, “there is a need to assess each case on its merits, rather than following rigid rules”. It is clear from an audit of handling of renal biopsies in the UK, performed in 1999, that standardised operative procedures vary widely, and that many laboratories fall short of “best practice”. A probable reason for this is that there is very little hard evidence to support any specific recommendations. In the UK audit, a questionnaire was circulated to all members of the UK Renal Pathology Group and returns were received from 50% of the 54 laboratories represented. It is interesting to compare current practices with Dr Furness’s guidelines.

Dr Furness recommended that all specimens should be examined in the biopsy room for adequacy, using a dissecting microscope. However, in only 15% of units this was performed as routine. Failure to confirm the presence of renal cortex in the specimen would be expected to increase dramatically the proportion of inadequate biopsies. This was not the experience in Manchester, however, where in 1994, as a result of staff shortages, the practice of sending an MLSO to attend every biopsy procedure was stopped. In Oxford, the histopathology laboratory is on a different site to the renal and transplant units; neither an MLSO nor a pathologist attends biopsies, as was once the case.

Furthermore, what constitutes an adequate specimen is difficult to define and to some extent depends on the nature of the pathology. More tissue is required to detect focal than diffuse lesions. This has been demonstrated in renal allograft biopsies; in the validation study of the CCGT classification of allograft pathology, those biopsies showing acute vascular rejection contained the diagnostic arteritic lesion in only one of two cores taken in 82% of cases. In the UK audit, it was found that 19% of renal tissue routinely taken varied from one to four in different centres. Dr Furness recommends that division of the specimen should be done within minutes of the biopsy being taken, to avoid artefactual ultrastructural changes. Although subtle subcellular changes do develop if fixation is delayed, for routine diagnostic electron microscopy (EM) rapidity of fixation is much less crucial.
excellent ultrastructural detail and is the feasible form of choice for EM in some laboratories. Occasionally, we have received specimens that have been stored unfixed in transport gel for two days, and found preservation to be adequate for the purpose of diagnostic EM.

There is also variation in the immunohistochemical techniques used when handling native renal biopsies. A frozen sample for immunofluorescence (IF) is taken routinely in 81% of laboratories; the remaining 19% rely entirely on immunoperoxidase (IP) stains performed on paraffin wax embedded sections. This, in part, reflects varying success in achieving reliable results with IF for immunoglobulins and complement. In the case of early transplant biopsies, only 30% of laboratories routinely take tissue for IF. In those that do, it is often for research purposes rather than for patient management. Similarly, most laboratories (88%) routinely take tissue for EM from native renal biopsies. Because some of the most common renal diseases, such as thin membrane nephropathy, can only be diagnosed ultrastructurally, those laboratories that do not take tissue for EM are certainly falling short of “minimum adequate practice”. Although it may be “best practice” to perform EM in all cases, it is probably sufficient to store this tissue as a resin block and only perform EM if the light microscopy is non-diagnostic. In many instances, EM will not influence patient management and the “minimum adequate practice” would, therefore, be to consider each case on its own merits and perform further investigations only if necessary. At present, EM does not have a clearly defined role in the assessment of early transplant biopsies and the UK audit found that only 38% of laboratories routinely take tissue for EM from these specimens.

The choice of which special investigations are performed should, at least in part, be determined by our clinical colleagues. Nephrologists differ widely in how aggressive they are in investigating patients with asymptomatic renal disease, such as those presenting with microscopic haematuria detected at a routine health check. In some centres a biopsy will only be performed if it is likely to affect management of that patient; in others, biopsy practice is partly driven by research interests. Equally, the information required from the pathologist will depend on its potential clinical value. For example, providing a measure of the severity of chronic tubulointerstitial injury in a patient with membranous nephropathy is of far more value to the nephrologist than knowing the glomerular disease stage, as defined by ultrastructural appearances.

In the UK audit, the number of paraffin wax sections routinely cut for native renal biopsies varied greatly—from two sections on two slides to two sections on ten slides—again reflecting a lack of evidence base. In his article, Dr Furness indicated that the number of sections that should be cut and examined depends on the nature of the question. A renal biopsy standard operative procedure should, however, include examination of sufficient sections to enable the diagnosis of conditions in which the pathology is usually focal. In the case of primary focal segmental glomerulosclerosis, this is considerably in excess of two. For renal transplant biopsies, the Banff classification recommends that at least three haematoxylin and eosin (H&E) and three periodic acid Schiff or methenamine silver stained sections should be examined. The rationale behind this is that the diagnostic lesions of acute rejection—tubulitis and arteritis—are often focal. A recent review of transplant biopsies in Manchester concluded that one third of diagnoses of acute vascular rejection would be missed if only one, rather than three, H&E sections were examined (GP McCarthy, ISD Roberts, 2000, unpublished data). All laboratories that handle renal biopsies should review their standard procedures, particularly if they do not conform to Dr Furness’s guidelines or “usual practice”, as indicated by the UK Renal Pathology Group audit. As the diagnostic questions asked by nephrologists change and new techniques emerge, procedures will inevitably require updating, and we will need to provide the evidence that any changes introduced are of demonstrable benefit to patient management.

2 Roberts ISD, Tarpey P. Use of a transport gel for antigen preservation in unfixed renal biopsies. J Pathol 1996;179:31A.

In reply

I am grateful for the opportunity to respond to the letter of Drs Roberts and Davies on the ACP Best Practice article “Renal biopsy specimens”, although they say very little with which I disagree. Most of their points of difference relate to “current practice” or “minimum adequate practice” rather than “best practice”. For example, the observation that electron microscopy (EM) can provide useful information even if fixation is delayed for a day or more is interesting and useful information. It supplements my observation that tissue from the paraffin wax block can be reprocessed for EM, but it does not alter the fact that best practice is to get the tissue fixed quickly.

The UK audit that they describe is a welcome update of a similar study that we performed in 1995 and which influenced the development of the ACP guidelines.

There is one small point where I think that Roberts and Davies misrepresent my suggestions. In their discussion of identifying and dividing the sample under a dissecting microscope, they imply that this has to be done by a pathologist or an MISO. We have found that nephrologists and radiologists can identify renal cortex and divide the biopsy appropriately with only minimal training. Again, rapid division is best practice; taking a bit longer is probably quite adequate in most circumstances, but (for example) in the future a delay will probably invalidate studies of gene expression.

Apart from these rather trivial quibbles I welcome Roberts and Davies’s contribution to the discussion.

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Book reviews


To my knowledge, this is the first comprehensive textbook dealing exclusively with the histological interpretation of needle core biopsy samples. To date, there have been one or two books edited by radiologists on needle core biopsy, which include chapters on histological interpretation. These by their nature have been restricted to basic principles.

The author of this book is of course well known to pathologists involved in breast disease reporting. He has numerous widely cited publications in peer review and in recent years has produced a major textbook on breast pathology based on his personal experience. Personally, I am a great admirer of his achievement, enthusiasm, and dedication to the field of breast pathology. For this reason, reading this book has been a pleasure.

First, I would point out that this book although dealing principally with needle core biopsy interpretation is also a distilled version of Rosen’s textbook of breast pathology. Diagnostic entities are described in succinct detail and are well referenced.

The book includes 31 chapters, the first seven dealing with normal anatomy and benign conditions, including one chapter on microcalcifications, which form diagnostic groups that appear to be gaining prominence, particularly in the American literature. Rosen recognises that most adenomyoepitheliomas are variants of intraduct papilloma and closely related to ductal adenoma and pleomorphic adenoma.

There is a substantial chapter on ductal hyperplasia and intraduct carcinoma, which covers in detail the difficulties of distinguishing the microfocal changes present in core biopsy. In this chapter the author recognises that there are some challenging forms of atypical ductal proliferation that exhibit prominent intraductal and architectural papillia, but retain the focal characteristics of usual type hyperplasia, and comments that some pathologists would ignore these latter features for considering the purchase of this book. First, the widespread option of needle core biopsy for diagnostic sampling of breast lesions clearly is the door for a textbook, such as this, which considers many of the diagnostic problems that are now being encountered. Second, it serves as an updated and concise version of Rosen’s major textbook. Those of you reporting breast disease who have not purchased this textbook could “kill two birds with one stone” by acquiring a copy of this book. I will be placing my copy in our reporting room and suspect that it will spend more of its time open on the bench top rather than gathering dust on our library shelf.


In this book, an impressive amount of different molecular techniques that can be used in vascular research are described in great detail.

In summary, methods of molecular biology are described related to gene isolation, characterisation, expression, and transfer, and (of course) cell death.

In each chapter, the principle of the technique is explained (of course a basic knowledge of molecular biology is necessary). Subsequent materials and methods sections are described stepwise. Each chapter ends with notes that give extra clues for doing the experiments, and also functions as a troubleshooting section. Also helpful are the illustrations of the outcome of the described experiments, when successful. In the last chapter, gene therapy protocols are described, according to recent developments in this field.

Although all these methods can be used in pathological specimens, for general histopathology it is probably less suitable. Nevertheless, it is a very interesting book and I recommend it strongly for researchers, including pathologists, who are doing research in the field of vascular disease.


I thoroughly enjoyed reading this compact, stimulating, and refreshingly thought provoking book. It really puts cancer into an evolutionary context. It was pitched just right for me; as for most doctors, even those involved with cancers and leukaemias, my knowledge of evolution, history, epidemiology, and molecular biology is very focused on and tends to be limited to what affects my daily practice. So, essentially, most of us are laymen. It is the sort of book that will be enjoyed by scientists, doctors, and many of those whose primary interests lie in the arts and the humanities, not to mention pathologists too.

This book, with its almost conversational tone, allows us all to follow the arguments in what are potentially impenetrable arenas with surprising ease. Some of that ease is probably an illusion, but a welcome one. One’s confidence in Mel Greaves to lead us through the jungle of cancer is probably as important as truly understanding the implications and fine detail of the paths and surrounding countryside through which he takes us. Just occasionally, one can end up at a conclusion really believing one understands how one got there, only on reflection to realise that one might need to read the argument all over again. Perhaps I should replace all the “ones” with “I”! It’s a seductive story, and well told too—that’s what carried me along, rather than my own intrinsic abilities to understand. But I do confess I got almost as much pleasure rediscovering how I got to some of his destinations as I had when I first arrived.

I O ELLIS

Human Adverse Drug Reactions

30 May 2001, Royal College of Pathologists, London, UK.

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Correction


In table 1 the time of the first sample should have been at −11, −7, and −4 days in patients 1, 2, and 3, respectively; similarly, in table 2 the time of the first sample should have been at −12, −6, −4, and −1 days in patients 1, 2, 3, and 4, respectively. The authors apologise for this oversight.